ONE HEALTH TOXICOLOGY: EXPANDING PERSPECTIVES AND METHODS TO ASSESS ENVIRONMENTAL CONTAMINANTS

By

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Abstract

The discipline of One Health is founded on the principal that environmental health, animal health, and human health are interconnected. Although the field has been largely focused on zoonotic diseases, examining concepts such as toxicology under a One Health lens can offer a more holistic and preventative approach to research and implementation and, in particular, how fish-based diets may be involved with One Health outcomes. Here we present three general case studies that demonstrate new approaches to investigating One Health toxicology. In Chapter One we show how Arctic canids can be used as environmental sentinels for human health. We discuss three separate canid studies; in the first we find that Arctic foxes can act as sentinels of Arctic contaminants due to their foraging plasticity, in the second we examine the use of fish-fed sled dogs as a model for the effects of a fish-based diet on contaminants exposure and gene transcription, and in the third we develop the sled dog as a model for particulate matter air pollution in the Fairbanks North Star Borough. In Chapter Two we utilize the Steller sea lion, a non-model organism, as a sentinel for the effects of fish-based diet mercury exposure induced whole-genome changes in gene transcription (RNA-Seq). Using newly developed informatics tools we assemble a *de novo* transcriptome and examine large scale changes in gene expression related to mercury exposure and other One Health uses. This approach is extremely adaptable and has the potential to be applied across numerous non-model organisms and contaminants. We also applied a microbial mining algorithm to our RNA-Seq data and found evidence for a hemotropic *Mycoplasma spp.* in one of our samples. In Chapter Three we examine sources of mercury exposure for pregnant women from La Paz, Baja California Sur, Mexico. We found mercury concentrations to be generally low among the examined fish species and staple foods. While typical dietary assessments rely on recall surveys and questionnaires, we found that examining chemical biomarkers of diet including stable isotopes of carbon and nitrogen are critical in dietary risk assessment. Taken together these three investigations offer valuable lessons and techniques which can be applied to the field of One Health toxicology; especially to those fish diet based systems.

Table of Contents

List of Figures

List of Tables

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"It is advisable to look from the tide pool to the stars and then back to the tide pool again."

John Steinbeck, *Log from the Sea of Cortez* (1951)

Chapter 1 - **One Health Toxicology – Review of concepts, tools and scope of an emerging discipline**[1]

[1] John Harley

1.1 One Health

One Health is an encompassing scientific collaboration founded on the concept that human health, animal health, and environmental health are inextricably linked (van Helden et al. 2013), and that the study of any part should be conducted with an eye toward holistic interconnectedness. As an integration of ideas from multiple disciplines One Health has gained traction due to the encouragement of data sharing, successes in areas of zoonotic diseases, and the proliferation of holistic environmental perspectives (The World Bank 2010, Buttke 2011, Kahn 2017, Coons 2017).

The areas that have seen the most development and research under One Health are examples of emerging zoonotic diseases (Figure 1.1). Jones et al. (2008) found that the majority (71.8%) of emerging infectious diseases (EIDs) in humans originated in wildlife, a proportion that appears to be increasing over time. In the case of EIDs, the intersection of environment, animal, and human health is profoundly tangible, and it is fruitful and necessary to consider the effects of a changing climate on the exposure of humans and animals to pathogens (Mills et al. 2010). Indeed, several researchers have noted that human exposure to novel zoonotic agents has resulted in several epidemics of vector borne diseases due to changing climate conditions which alter the ranges of normal hosts and reservoirs of these pathogens (Daszak et al. 2001, Greer et al. 2008, Lindgren et al. 2012).

A strong movement occurred during the 1990-2000s towards increasing communication and collaboration between health-related disciplines. This was a period when several highly contagious emerging diseases including avian flu (Ferguson et al. 2004) and severe acute respiratory syndrome (SARS, Guan et al. 2003) were causing illness and deaths around the world. Both of these diseases were considered to be zoonotic in origin, and the questions of how, where, and why these diseases were transmitted to humans became critical unknowns to explore for human health. Research has suggested that several risk factors including socio-economic status and land use practices are associated with "hot-spots" of zoonotic disease transmission globally (Jones et al. 2008). It has also been suggested that climate change is likely to alter human-animal-pathogen interactions (Hueffer et al. 2011, Lindgren et al.

2012, Engering et al. 2013), although it should be noted that the increasing rate and extent of global movement of humans and domesticated species has the potential to facilitate novel exposures to pathogens independent of a changing climate.

1.2 One Health Toxicology

While EIDs have received the preponderance of attention under One Health, biotoxins, heavy metals, and other contaminants also represent threats to human, animal, and environmental health. Changes in environmental conditions and human development patterns might alter exposures of both humans and wildlife to natural and anthropogenic toxicants. The implications of these changes for human and animal exposure to environmental contaminants are presented as a conceptual model in Figure 1.2.

1.2.1 Sentinel Species Roles

Some researchers have pointed out that the role of toxicants in One Health collaborations can benefit from increased communication and development (Beasley 2009, Buttke 2011). While the movement of contaminants in an ecosystem is often complex and multifaceted, the linkages of environmental, animal, and human health are readily apparent and can often be investigated through the use of sentinel species.

The concept of a sentinel species is not novel, and their use has been and should continue to be embraced by One Health toxicology (Stahl 1997). Both wild and domestic animals have been utilized as sentinel species and offer different perspectives for examining human risk. Domestic animals (such as dogs, *Canis lupus familiaris*) share similar environments to their human owners, and are often exposed to similar contaminants via air, water, and occasionally diet (Backer et al. 2001, Dunlap et al. 2007, Reif 2011, Harley et al. 2016). Additionally, wildlife species can provide integrated information about the availability, concentration, or transport of contaminants affecting a particular ecosystem (Basu et al. 2007, Bossart 2011), especially for apex predators (Sergio et al. 2008). Often these top predators, utilized as sentinel species, are monitored for accumulation of contaminants from multiple environmental niches (i.e.

pelagic, near shore, benthos) negating the need to sample a variety of species from each of these niches. Sentinel species can provide important information regarding both wildlife and human exposure to contaminants and these data can be interpreted for exposure risks or toxic effects without having to consider human's predilection for voluntary contaminant exposures (e.g. smoking, alcohol, or other confounding variables, Dunlap et al. 2007).

Around the turn of the 20th century coal miners began using domestic canaries (*Serinus canaria domestica*) to detect dangerous concentrations of carbon monoxide in poorly ventilated shafts. Canaries are particularly sensitive to carbon monoxide due to their respiratory physiology and served as an early warning system for the more carbon monoxide resilient humans. Although in some systems the use of animal sentinels was already in practice, over the next several decades there were several catastrophic instances of human exposure to contaminants that might have been avoided or ameliorated by strategically surveying and examining animal sentinels (e.g. "preventative One Health"). One such event was the Minamata Bay disaster in Japan, in which large quantities of methylmercury waste were released into Minamata Bay (from 1932-1968), causing the death of over 1,700 people to date (Ministry of the Environment, Japan). Symptoms in cats of severe neurological disease were recognized years before the first cases of methylmercury poisoning were described in humans; clinical presentations and outcomes of erratic behavior, ataxia, and death caused the disease to be known as the "dancing cat disease" (Rabinowitz et al. 2010, Buttke 2011). Both humans and cats were exposed to methylmercury through the consumption of fish from the contaminated bay (Clarkson and Magos 2006, Rabinowitz et al. 2010). Another event occurred in Missouri, USA in 1971 when several horse arenas were sprayed with a mixture of chemical waste and oil which contained high concentrations of dioxin (Reinhold 1983). In the days following spraying events workers raked up the carcasses of dead sparrows, followed shortly by the deaths of cats, dogs, and horses (Sun 1983). Several children became ill after playing in the dirt in the arena. The town was evacuated during a flooding event in 1982, and following soil testing by the Environmental Protection Agency (EPA) and Center for Disease Control (CDC), it was recommended

that the town of Times Beach not be re-inhabited until after the flood had subsided, due to high concentrations of the chemical (Sun 1983).

Today the use of sentinel species to study environmental toxicology has expanded in scope, and researchers have increased their vigilance in order to avoid situations like Minamata Bay or Times Beach. As presented in Chapter 2 of this thesis, Harley et al. (2016) showed that sled dogs (*Canis lupus familiaris*) in Alaska were a useful biomedical (e.g. gene expression) model to study the effects of particulate matter ($PM_{2.5}$) exposure. Grove et al. (2009) outlined data examining ospreys (*Pandion haliaetus*) as sentinels for human exposure to contaminants from aquatic ecosystems across North America. Jessup et al. (2004) has advocated the use of the sea otter (*Enhydra lutris nereis*) as a sentinel for human exposure to marine based contaminants and biotoxins due to sea otters' generally small home range and prey items that overlap with the marine diet of coastal human populations. Some pinnipeds (i.e. harbor seal, van Hoomissen et al. 2015) can act as sentinels for marine contaminants via a fish-based diet. This concept is explored in detail in the third chapter of this thesis.

1.2.2 Biotoxins in One Health

An increasingly important example of the interactions within the triad of One Health are the increasing occurrence, density and severity of harmful algal blooms (HABs) (Moore et al. 2008, Paerl and Huisman 2009). HABs are caused by a proliferation of cyanobacteria, diatoms, or dinoflagellates that produce bioaccumulating toxic secondary metabolites. While the conditions favoring the proliferation of HABs are still being elucidated, the organisms producing these biotoxins seem to favor warmer water, and initial reports have suggested that increasing concentrations of dissolved CO_2 may increase the production of toxins (i.e. saxitoxin) in dinoflagellates (Fu et al. 2012). Both of these changing conditions are predicted under current climate models. Shifts in ocean temperatures might favor the range expansion of HAB forming species, which could lead to the exposure of potentially sensitive populations and systems to a novel toxin. For instance, until recently there were few reports of saxitoxin (paralytic shellfish poisoning) or domoic acid (amnesic shellfish poisoning) in marine mammals foraging in Alaska.

However, a recent report found both of these toxins prevalent in several Alaska marine mammal species at potentially harmful concentrations (Lefebvre et al. 2016). The impact of toxins produced by HABs are profound and have been implicated in mass die offs of birds, fish, and mammals (White 1977, Van Dolah 2000, Shumway et al. 2003). Several of the toxins produced by HABs accumulate in toxic concentrations in filter feeders, such as shellfish, important food items for some mammals and humans (Hallegraeff 1993, García et al. 2004). Both domoic acid and saxitoxin have been known to cause acute poisoning events in humans following the consumption of contaminated shellfish (Hallegraeff 1993, García et al. 2004).

1.2.3 Heavy Metals and Persistent Organic Pollutants in One Health

Of course, biotoxins are not the only poisons that humans and wildlife are exposed to in the marine environment. Some heavy metals (i.e. mercury (Hg) or cadmium) and anthropogenic chemicals (i.e. polychlorinated biphenyls, PCBs) are present in nearly all marine environments, and many can bioaccumulate and biomagnify in appreciable and toxic concentrations in humans and wildlife. The interactions of some of these chemicals with respect to human and wildlife health is demonstrably different than the case of biotoxins because (1) their temporal and spatial distribution is generally chronic and widespread, as opposed to acute localized HABs, and (2) their occurrence in marine systems often involves human activities with complex oceanographic, atmospheric, and biological transport and dispersion (Burek et al. 2008). Despite the natural occurrence of heavy metals their availability to biota has increased from direct (e.g. mining) and indirect (e.g. release from burning fossil fuels) uses of natural resources.

The prediction of climate-animal-human health interactions with heavy metals and anthropogenic pollutants is complicated. Humans, while simultaneously subject to the effects of pollutants, are also the releasers (mobilizers), manufacturers and transporters of some contaminants. Increasing use of the Arctic for shipping, resource development, increased human habitation, and agriculture is likely to alter both animal and human exposure to a number of industrial pollutants (Burek et al. 2008). In the context of

infectious diseases, the climatically driven movement of a parasite or pathogen can be of concern for animal or human health by exposing naïve or sensitive populations. However, with respect to industrial pollutants, the increased mobilization of human populations will alter exposure regimes in the coming decades.

The changing climate also has the potential to affect toxicant exposures in a more passive way. Global transport of volatile organic contaminants is driven by air and water transport (Simonich and Hites 1995), and changes in precipitation patterns and sea ice extent are likely to alter transportation patterns of contaminants to the Arctic (Dore 2005, Screen and Simmonds 2010). The role of sea ice in contaminant exposure is being examined, as persistent chemicals and metals that were deposited in relatively inert "sinks" (i.e. ice and tundra) are being revolatilized with the warming of the Arctic (Ma et al. 2011).

1.3 Emerging Tools

1.3.1 Communication and Data-sharing

Since One Health represents a collaborative organization of data and perspectives rather than a cutting edge specialized biomedical field, it is not surprising that new tools and techniques applied to One Health related projects are often developed and validated in other fields. Indeed, one of the challenges of One Health is the application of modern methods and informatics in ways that can be interpreted and communicated in a holistic context. In some cases, rapid dissemination to managers and decision makers is critical for optimal use of One Health based information gathering. As some have pointed out, the goals of One Health necessitate successful communication with the public, and studies which solely report the prevalence of zoonotic diseases or which don't adequately convey relevant findings fall short not only in with respect to scientific communication but also within the context of preventative One Health.

Some of these emerging tools aim to encourage and streamline data sharing among disciplines, while others have generated networks for collaboration and reporting. For instance, in 2016 the CDC launched the One Health Harmful Algal Bloom System (OHHABS) which collects data from state and

territorial health departments regarding occurrence of HAB events or human and wildlife illness following consumption of contaminated shellfish. Other research has been aimed at developing novel, less-invasive, and unbiased surveillance techniques for monitoring infectious diseases in wildlife populations (Reed et al. 2014).

1.3.2 DNA/RNA Sequencing

Undoubtedly, one of the greatest recent breakthroughs in biology-related fields was the advent of high-throughput DNA sequencing technologies. Since becoming accessible in the early 2000s, next-generation sequencing tools (NGS) have increased the speed, accuracy, and scope of genomic sequencing, all while reducing the cost to researchers to fractions of a penny per megabase (Mb) (Goodwin et al. 2016). While these technologies and methods were developed on the frontiers of molecular biology and engineering, their prevalence and availability have facilitated their incorporation into new disciplines. For instance, the shotgun sequencing by NGS of complimentary DNA (cDNA) libraries known as RNA-Seq has been utilized in toxicology as a tool for examining genetic response to a particular contaminant (Mortazavi et al. 2008, Morey et al. 2016). These technologies have led to the development of biomarkers, discovery of metabolic and toxicological pathways, as well as the bioinformatics-based assessment of transcriptomic effects of novel contaminants. NGS has several advantages over targeted sequencing, including lower cost per Mb and a relatively unbiased screening method compared to targeted approaches such as quantitative polymerase chain reaction (qPCR).

1.3.3 Informatics

While RNA-Seq has been applied to numerous laboratory animals, the application of RNA-Seq to assessing transcriptomic profiles of wildlife species is challenging. Aside from the typical problems of working with wildlife populations such as logistics of fieldwork, and balancing sufficient sample sizes against disruption of the animals, there are a number of challenges unique to the application of RNA-Seq to wildlife populations. First, RNA is an inherently unstable molecule, and the degradation of RNA has

been shown to influence gene expression studies from microarrays (Opitz et al. 2010). Thus, high quality RNA is essential for comparisons of gene expression, especially given a small sample size. Second, there are fewer genomic resources available for non-traditional model organisms. For instance, at the time of this writing, there are only 13 gene sequences for the Steller sea lion (*Eumetopias jubatus*), all of which are mitochondrial. The closest related species to the Steller sea lion that has a complete genome available is the Pacific walrus (*Odobenus rosmarus divergens*), whose last common ancestor with the Steller sea lion occurred approximately 35.7 million years ago (Higdon et al. 2007). Despite this, Chapter 3 of this thesis utilizes RNA-Seq to expand the biomedical toolbox for Steller sea lion research in a One Health context. We show that RNA-Seq can be used for assessing toxicants and blood RNA samples can be examined for presence of known or possible pathogens.

In spite of the hurdles, the potential applications of NGS to assist the goals of One Health toxicology are immense and achievable. Environmental toxicology has for years used gene expression as a biomarker of a response to a particular contaminant or stressor, and the ability to sequence entire transcriptomes has revealed complex gene responses in both model and non-model organisms (Schirmer et al. 2010, Garcia et al. 2012, Huang et al. 2012). Many of these have been linked to phenotypic responses (e.g. biotransformation, adverse effects). cDNA microarrays were among the first of these high throughput technologies to succeed in ecotoxicology (Neumann and Galvez 2002), and many researchers are continuing to make use of this technique (Liu et al. 2013). However, RNA-Seq has several advantages over microarrays, including (1) increased detection of rare transcripts, (2) identification of genetic variants, and (3) broader dynamic range to detect highly differentially expressed transcripts (Zhao et al. 2014), as discussed in more detail in Chapter 3. These advantages have led some to suggest that the microarray will soon be supplanted by RNA-Seq in ecotoxicology, although due to the relative lack of genomic information for wildlife species, it may be several years before the microarray technology disappears entirely.

1.4 Expanding the Scope of One Health Ecotoxicology

One Health is an expansive and encompassing perspective, and there have been a number of advances within the field that have contributed to continued success. Yet one of the greatest benefits of advancing One Health is the fostering of communication among focus areas (collaboration), including both traditional disciplines within the triad (human health, animal health, environmental health) as well as areas of research which are not traditionally considered applicable to One Health, but which can offer tools and techniques that will benefit One Health research. As mentioned earlier, bioinformatics reliant tools such as RNA-Seq and microarrays have been adapted from their original usages in biomedical model organisms to examine the effects of different environmental stressors on wildlife (Mancia et al. 2012, Liu et al. 2013). In addition to biomedical techniques, traditional ecological tools can be used in a One Health context.

Chemical feeding ecology analyses (i.e. stable isotopes of C and N) have been utilized in ecological research to answer questions about trophic ecology and diet and are now being used to assess questions of potential contaminant exposure of humans and wildlife through diet (Rea et al. 2013, Bentzen et al. 2014). This approach, which we have applied to fish consumers in Mexico in Chapter 4, benefits from integration of complex biomedical information with aspects of innovative biostatistics and models for assessing major and minor drivers of exposure to toxicants. This chemical feeding ecology of Hg in this region is supported by ongoing efforts with other graduate students in Mexico using species from multiple trophic levels (urchins to sharks). Indeed, one of my career objectives is to work in this region to make these One Health linkages to better address public health and management of marine environments.

1.5 Following Chapters

In this dissertation, I present three research chapters aimed to expand the scope of One Health toxicology by introducing new techniques and perspectives. The incorporation of techniques developed in other fields such as RNA-Seq and chemical feeding ecology can work to expand the scope of One Health,

especially within the context of toxicology (Figure 1.3). A large portion of this effort has been to address fish consumption based systems in a One Health context. Chapter 2 shows how sled dogs and other canids can be used as molecular toxicology models and sentinels for environmental contaminants in the Arctic and subarctic. I then demonstrate how Steller sea lions and other wildlife populations can be assessed for transcriptomic responses to contaminants, as well as a parallel method screening for the presence of potential and known pathogens (Chapter 3). Finally, I demonstrate how C and N stable isotopes feeding ecology can explain variation in human exposure to mercury and address likely sources of exposure and overall diet composition (Chapter 4). Taken together, these three studies have helped to adapt new tools and methods to the field of One Health toxicology and assisted in the furthering of animal, human, and environmental health.

1.6 Figures

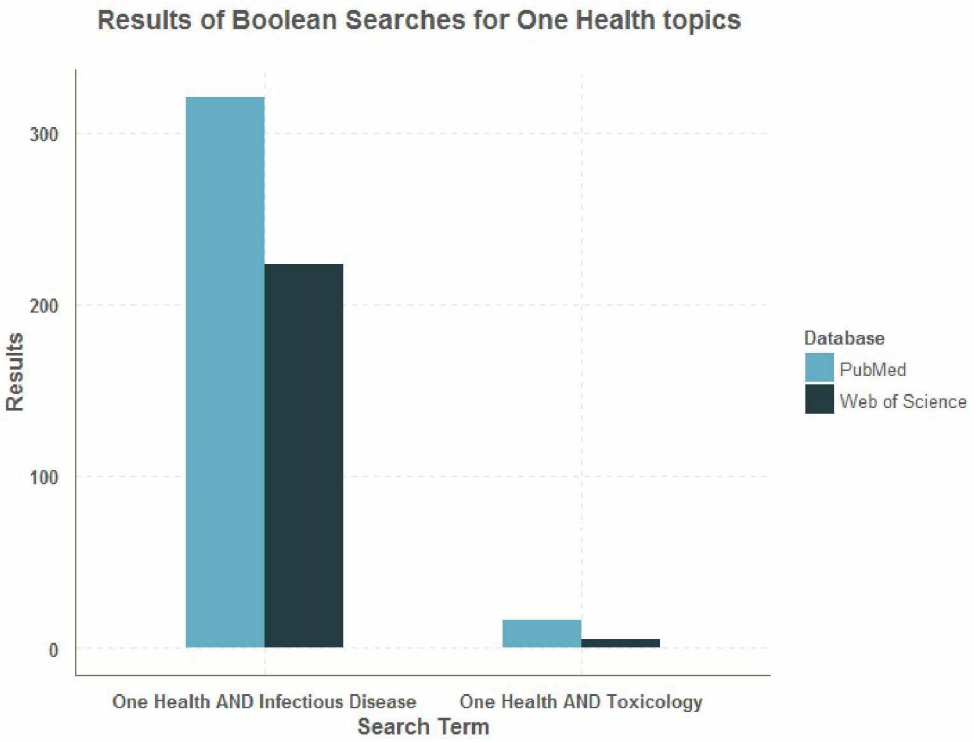

Figure 1.1 - Compared to infectious disease, toxicology has received relatively little attention in One Health. Search conducted in August 2017 and encompassed publication years 1990-2017.

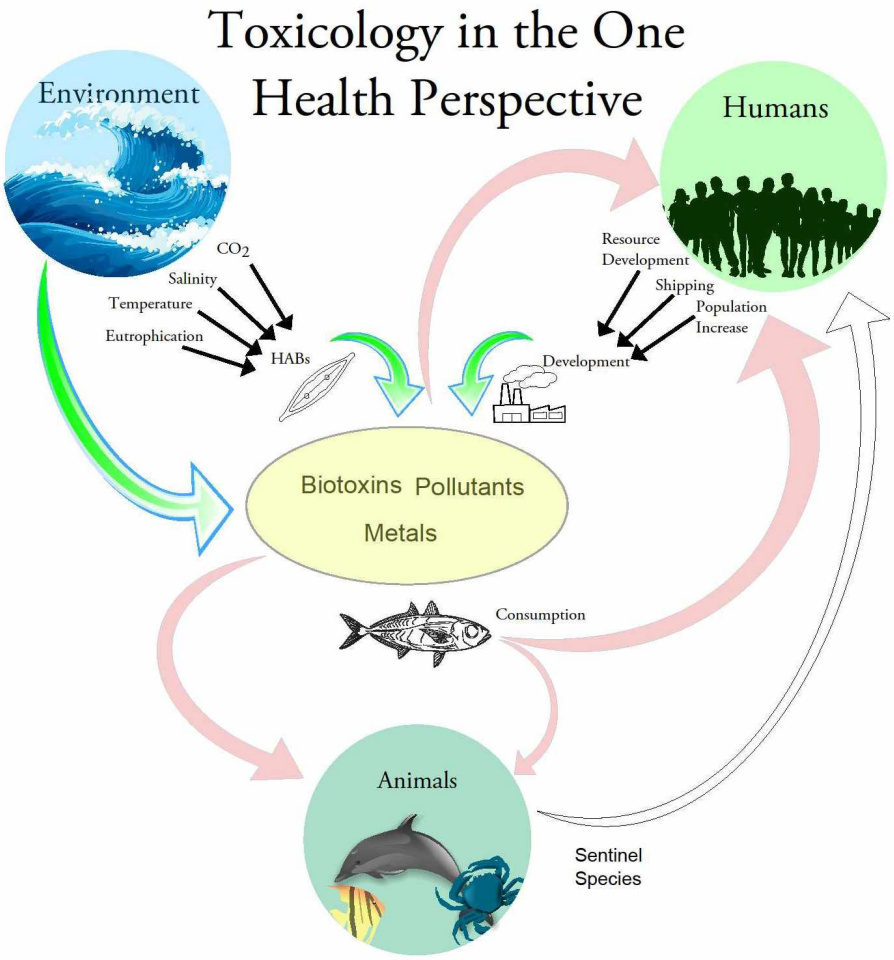

Figure 1.2 - Conceptual model of toxicology in the One Health perspective. Climate change is likely to alter human and animal exposure to biotoxins such as those produced by harmful algal blooms (HABs). Similarly, increasing human development and expansion will alter production and exposure to heavy metals and organic contaminants.These contaminants affect both animal and human health.

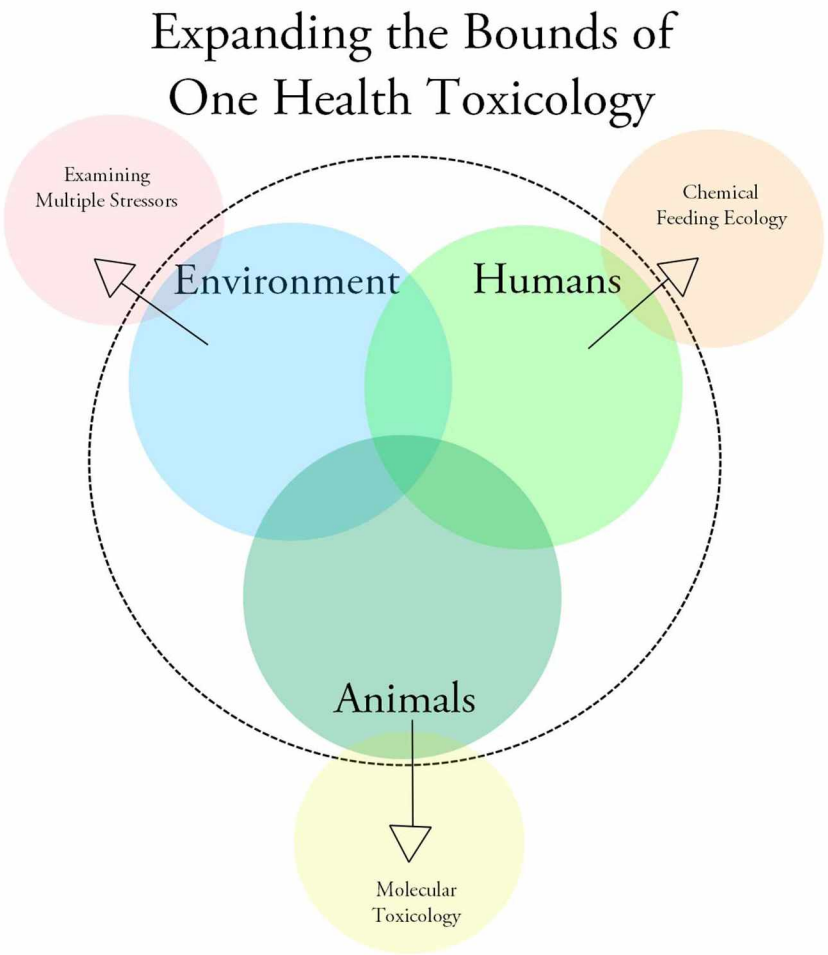

Figure 1.3 – Conceptual diagram illustrating an overview of this dissertation. In the three research chapters presented here we expand the scope of One Health toxicology by adapting tools and techniques from adjacent fields such as molecular biology (transcriptomics, pathogen mining), chemical feeding ecology (C and N stable isotopes), and the impact of multiple environmental stressors (air pollutants, mercury in seafood) across diverse taxa (canids, pinnipeds, and hominids).

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Chapter 2 - **Using domestic and free ranging Arctic canid models for environmental molecular toxicology research**[2]

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2.1 Abstract

The use of sentinel species for population and ecosystem health assessments has been advocated as part of a One Health perspective. The Arctic is experiencing rapid change including climate and environmental shifts as well as increased resource development, which will alter exposure of biota to environmental agents of disease. Arctic canid species have wide geographic ranges and feeding ecologies and are often exposed to high concentrations of both terrestrial and marine-based contaminants. The domestic dog (*Canis lupus* familiaris) has been used in biomedical research for a number of years, and has been advocated as a sentinel for human health due to its proximity to humans and, in some instances, similar diet. Exploiting the potential of molecular tools for describing the toxicogenomics of Arctic canids is critical for their development as biomedical models as well as environmental sentinels. Here we present three approaches to analyzing toxicogenomics of Arctic contaminants in both domestic and free-ranging canids (Arctic fox, *Vulpes lagopus*). We describe a number of confounding variables that must be addressed when conducting toxicogenomics studies in canid and other mammalian models. The ability for canids to act as models for Arctic molecular toxicology research is unique and significant for advancing our understanding, and expanding the tool box for assessing the changing landscape of environmental agents of disease in the Arctic.

2.2 Introduction

Sentinel species serve as early warning indicators of ecosystem conditions as part of the "One Health" paradigm which utilizes a holistic approach to disease management in ecosystems, wildlife, and human populations. Some apex mammalian predators and omnivores are commonly utilized as sentinel species because of their relatively long life spans, subcutaneous fat stores which accumulate lipophilic anthropogenic contaminants, and accumulation of some non-lipophilic contaminants in relatively easily accessible matrices such as hair. [1] The Arctic Monitoring and Assessment Program (AMAP) region (henceforth "Arctic", Figure 2.1, www.amap.no) contains populations of both marine and terrestrial mammals, some of which have value as environmental sentinels. [2,3] While it is difficult to generalize risk assessment of contaminants across species or geographic ranges, several studies have indicated that concentrations of some contaminants (i.e. mercury (Hg) and some polychlorinated biphenyl (PCB) congeners) are above thresholds of concern or correlate with potential adverse effects in some Arctic populations or sub-populations (see reviews [4–6]). Increasing environmental variability, due in part to climate change and human development, will undoubtedly influence contaminant exposure, body condition, and pathogen exposure.[7,8] The health effects of contaminants in this context has not been well explored, thus making it increasingly prudent to develop tools to assess the health of individuals, populations, and ecosystems of northern latitudes.

Adverse effects concentrations and thresholds for toxic contaminant exposure are most often derived in controlled laboratory settings as solitary agents. In natural settings, exposure to complex mixtures can make the derivation of thresholds of adverse health effects in wild populations challenging, especially with respect to environmentally relevant mixtures of contaminants. However, using molecular toxicology methods, including assessments of mRNA populations (i.e. all mRNA molecules present in a sample), it is possible to detect biological responses at low exposure concentrations that provide insight on specific biochemical pathways involved. [9] Although linking changes in gene expression to higher levels of biological organization (i.e. adverse outcome pathways (AOPs)) is often challenging, these data

can assist with descriptions of mechanistic pathways of biotransformation and potential injury from novel and emerging contaminants and they also provide documentation of a biological response. Many Arctic predators (including humans) have relatively high concentrations of some inorganic and organic environmental contaminants (e.g. Hg, PCBs), and are thus suitable for studying the molecular toxicology of environmentally relevant concentrations of chemical mixtures. [4,10]

The Arctic contains populations of free-ranging canid species such as the Arctic fox (*Vulpes lagopus*), red fox (*Vulpes vulpes*), coyotes (*Canis latrans*), and the gray wolf (*Canis lupus*). Some populations of Arctic foxes and gray wolves have been shown to have extremely variable diets and, depending on location and season, can reflect components of both marine and terrestrial ecosystems. [11–13] The consumption of marine derived nutrients is associated with increased concentrations of mercury (Hg), and elevated concentrations of Hg have been found in both foxes and wolves due to an increase in marine-based foraging. [11,13] This enhances their utility as sentinels, especially for coastal human inhabitants with similar mixed utilization of terrestrial and marine resources. Marine and freshwater derived diet items, including diadromous salmonids, represent an important component of Arctic human and canid diets and are key components for contaminants pathways across the landscape. Due to their circumpolar distribution and previous use as biomedical models, the Arctic fox represents a valuable sentinel for Northern contaminant monitoring. [14–16]

In addition to free ranging canids, many northern communities (especially in areas of Alaska and Canada) are home to large teams (kennels) of sled dogs. Operators of these kennels often use locally derived diets that are similar to the resident human populations. [17] The sled dog has previously been utilized as a biomedical model in controlled feeding experiments examining the metabolism and physiological effects of exposure to organohalogens via whale blubber, which is an important subsistence item in many Arctic communities. [18–20] . Sled dogs do not engage in some activities that often complicate epidemiologic toxicology studies (i.e. tobacco and alcohol use) and they represent diverse trophic feeding levels and feeding ecologies (omnivory), which often mirrors the cohabiting human populations. [17]

Moreover, these dogs are housed in immediate vicinity to their handlers, and are thus also mirroring non-dietary environmental exposures such as airborne particulate matter (i.e. wood smoke). Particulate matter (PM) from vehicle emissions, wood stoves, and other combustions sources can become elevated during cold weather, especially in areas susceptible to temperature inversions such as Fairbanks, Alaska (USA).[21] Elevated PM concentrations have been implicated as a causative or exacerbating factor in a number of diseases that involve oxidative stress, alterations in DNA methylation and damage, and cellular injury, and have been correlated to a number of clinical disease states. [22] Sled dogs serve as excellent models for PM exposure and toxicogenomics studies for a number of reasons; (1) they are housed outdoors, eliminating the indoor/outdoor exposure gradient, (2) most animals are under similar kennel-wide diet and exercise regimens, (3) pedigrees are known for several generations, allowing for post-hoc gene-environment interaction analyses, and (4) they can be easily assessed for the interaction between diet and environmental stressors, such as ultraviolet (UV) exposure or air pollution that is similar to humans in the area.

Environmental contaminants from dietary and airborne sources, while often utilizing different exposure and detoxification pathways, can nonetheless lead to similar general mechanisms of cellular injury such as oxidative stress. Inducible molecular mechanisms exist that can protect against or repair oxidative damage, [23] and many utilize sulfur (S) or selenium (Se) based systems. Some S-based antioxidants such as glutathione (GSH), metallothionein, thioredoxin reductase (TXNRD), are induced or activated by oxidative stress. [24,25] Inducible cellular defenses to oxidative stress, such as those coordinated by the Nrf-1/Nrf-2 binding to antioxidant response elements (ARE), which have been shown to respond to a wide array of environmental stressors including cigarette smoke, environmental toxicants, and other inflammatory stressors. [26] Many toxicogenomics studies compare differences between pre- and post-treatment expression (i.e. Bouwens et al. [27]) however this study design may not account for stochastic and time dependent changes in gene expression.[28] For instance, Robinson et al. found that in mice, following exposure to 6 mg/kg $MeHg^+$, a number of genes that were upregulated at 8 hours were not upregulated 12

hours post-exposure, and thus concluded that $MeHg^+$ induced changes in gene expression may occur in a time-dependent manner. [29] Indeed, the lag in sampling following contaminant exposure, chronic or acute, may account for some of the variation seen within and between toxicogenomics studies (Figure 2.2). Long term studies of changes in gene expression due to diet or contaminant exposure are rare, and studies involving lower exposure concentrations (i.e. environmentally relevant) such as those encountered via dietary and occupational exposure, may benefit from long time scale examinations of changes in gene expression.

We present three scenarios evaluating the utility of Arctic canine models to describe gene expression in response to exposure to environmental contaminants. We demonstrate that (1) domestic sled dogs have time-dependent transcriptomic changes as a result of a 50% piscivorous diet (compared to 0% fish diet), (2) gene expression of free-ranging foxes can be reliably analyzed using a domestic canine microarray and these data can be used to model toxicant interactions of an environmental mixture of PCBs and Hg, and (3) domestic sled dogs can be effective models for transcriptomic changes caused by exposure to air pollution as measured by PM. We investigate the boundaries of the Arctic canid model, including key caveats of study design and limitations of extending this model in a One Health context and provide evidence that, despite difficulties in sampling associated with free ranging species and cold climates, samples collected in the field are of good quality for high throughput molecular toxicology analyses. We also examine the potential use of a commercially available canine microarray for use in an Arctic fox model using strict quality assurance measures (cross species validation). Further, our study emphasizes potential uses of transcriptomics analyses in canid sentinel species to evaluate the response to several toxic agents including airborne particulate matter, mercury via fish consumption, and diets with chemical mixtures containing PCBs and Hg.

2.3 Experimental design

2.3.1 Fish fed sled dogs

The general study design and Hg findings for this study are described in full in Lieske et al.[30] Briefly, eight sled dogs were randomly assigned to two feeding groups; a control group which was fed commercial fish meal-free dog kibble (*n=4*) (Standard Choice 26% Value Meal Dog Food, Fromm Family Foods, Mequon, WI) and a fish-fed group (*n=4*) which was given a 50% fish diet (one meal of chum (keta) salmon (*Oncorhynchus keta*), the other a meal of kibble each day; details of diet composition are described in Lieske et al.[30] Each treatment group consisted of 2 male and 2 female animals. Blood samples were collected via venipuncture from each dog two weeks prior to the controlled feeding, as well as once weekly during weeks 0-4 and once bi-weekly during weeks 6-12.

In order to compare gene expression between the two groups over the course of the 13-week experiment, a repeated measures ANOVA was utilized in which gene expression was compared between treatment groups with error nested in individual:week interactions. In order to eliminate differences in expression based on intrinsic differences between individuals (i.e. differences in gene expression pre-treatment), expression values are reflected as fold change difference from Week 0.

Equation 2.1

$$\frac{Gene\ \text{expression}_{Week\ n} / GAPDH\ expression_{Week\ n}}{Gene\ \text{expression}_{Week\ 0} / GAPDH\ \text{expression}_{Week\ 0}} = \text{Fold Change for Week } n \text{ over Week } 0$$

2.3.2 Mixtures of Hg and PCBs in Arctic foxes

Twelve Arctic foxes were captured during July 2007 using Tomahawk live traps baited with canned tuna in oil, placed outside of den sites and fox travel sites in the Deadhorse/Prudhoe Bay area, Alaska. Animals were sampled to study the health of free-ranging Arctic foxes including an assessment of disease and contaminants (approved by the University of Alaska Fairbanks Animal Care and Use

Committee, IACUC 07-20 and 07-30). Animals were placed in a squeeze trap for injection of immobilants via hand syringe [~ 6 mg/kg ketamine / 3 mg/kg xylazine, (20 mg of ketamine /10 mg of xylazine for 2.3 to 3.6 kg (5 to 8 lb) fox)] intramuscularly in the hind leg. Mass, sex and body morphometry were recorded. A cursory physical examination (description of pelage, teeth wear, scars, injuries, etc.) was performed by a veterinarian to identify any health issues. Blood (approximately 10-14 ml) was directly collected from the jugular vein (cephalic vein and femoral artery as backup) using a 21g ¾ inch needle blood collection set ("butterfly"). Blood volume draw did not exceed 1% of total body weight. After recovery from immobilants, animals were released back to the wild at their site of capture.

Of the animals captured, eleven Arctic foxes were selected for this study and the Hg and PCB levels had been determined for each animal (Table 2.1). We used two different strategies to analyze the microarray data. Our first approach divided the animals in groups based on the levels of Hg or PCB measured in tissue. This resulted in two discrete groups each of "low" (blood 5.5 ppb – 22.1 ppb) and "high" (blood = 26.6 – 55 ppb) THg, and low (below detection – 0.77 ppb) and high (2.47-4.69) $\sum$(40)PCBs. Genes with statistically significant differential expression in the low versus high Hg or PCB groups were identified using the Bioconductor limma package. P-values were calculated with a modified t-test in conjunction with an empirical Bayes method to moderate the standard errors of the estimated log-fold changes. P-values were adjusted for multiplicity with the Bioconductor package qvalue, which allows for selecting statistically significant genes while controlling the estimated false discovery rate. A limitation of dividing animals into groups of low and high Hg or PCB levels is that there is a considerable range of Hg or PCB concentrations and it is somewhat arbitrary to define which animals are designated "low" or "high". Furthermore, some animals with low Hg have relatively high levels of PCB and vice versa. Given that the samples were derived from Arctic foxes living in the wild, it was not possible to control Hg and PCB levels. Therefore, we employed a second analysis strategy that didn't require assignment of animals to discrete groups of low and high Hg/PCB levels but rather treated Hg and PCB levels as continuous variables in the linear model for gene expression.

2.3.3 PM air pollution study

A detailed description of $PM_{2.5}$ (particulate matter with diameter less than 2.5 μm) characterization and sampling design is presented in Montrose et al. [31]. Briefly, three mushing kennels were selected, with two kennels located within the Environmental Protection Agency's (EPA) $PM_{2.5}$ non-attainment boundary established for the Fairbanks North Star Borough (FNSB) in 2009. The third kennel was located ~30km outside of the Environmental Protection Agency (EPA) non-attainment area for $PM_{2.5}$. The three kennels had varying concentrations of $PM_{2.5}$ throughout the course of the study period (November 2012 – March 2013).[31] The 3-week period immediately preceding the blood sampling effort (January 26 – February 15, 2014) was used to determine that Kennel C had significantly lower $PM_{2.5}$ concentrations than both kennel A and kennel B. For each kennel 28-30 dogs were sampled. Dogs from kennel C were assigned to the "Low Exposure" group (*n=28*), and dogs from kennel A and kennel B were combined into the "High Exposure" group (*n=59*). Blood samples were collected via jugular or cephalic venipuncture. Hematological variables were measured by the UAF Animal Resource Center using DriChem® 4000 Chemistry Analyzer (Heska, Colorado, USA) and CBC-Diff™ Veterinary Analyzer (Heska, Colorado, USA).

Normality of quantitative hematological variables (presented in Montrose et al. [31]) and gene expression data were analyzed using Shapiro-Wilk test. Variables that were non-normally distributed were log_2 transformed. Some variables were non-normally distributed after log_2 transformation; these variables were excluded from ANOVA analysis and instead assessed by non-parametric analysis using the Mann-Whitney rank sum test. A one-way ANOVA was used to assess differences between exposure groups (high vs. low) and significant differences between response variables were assessed using Tukey's HSD. In order to investigate potential associations between gene expression and hematological variables, simple linear regressions were conducted with quantitative hematological variables serving as the independent variable and gene expression as the dependent variable. Outliers were examined using the Bonferroni inequality for computing p-values for studentized residuals. Observations with adjusted p-

values of $p<0.01$ were determined to be outliers and removed from regression analysis. Normality of residuals was examined for all regressions using q-q plots.

2.3.4 General methods

2.3.4.1 RNA preservation and extraction

All blood samples destined for molecular analyses were collected in PAXgene (Qiagen) RNA tubes and stored on ice in the field until they were placed in -20°C or -80°C freezers. RNA was extracted using PaxGene Blood miRNA Kit (Qiagen) and quantity and integrity determined using an Agilent 2100 Bioanalyzer (Agilent Technologies) or a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific).

2.3.4.2 RNA quality assurance

RNA integrity within the cell is dependent on a complex series of responses that are set in motion in response to insult (prior to or concurrent with sampling), including the critical interval between the time of collection and time of tissue preservation (e.g. stabilization of mRNA). Mechanisms of RNA degradation are complex and varied, and not necessarily consistent across the population of mRNAs. Thus, multiple measures of RNA and assay quality are required, especially under conditions where novel applications are being attempted such as sampling in extreme field conditions (captured Arctic fox on the coast of the Arctic Ocean; remote dog kennels).

RNA samples were quantified by measuring OD_{260} using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). RNA purity was assessed by measuring $OD_{260/280}$ (protein contamination) and $OD_{260/230}$ (contamination with organics) ratios. All samples had $OD_{260/280}$ between 1.8 and 2.2. RNA integrity was assessed with a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, California) using the RNA Pico kit. RNA integrity numbers (RIN) were calculated for each sample. The High Pure miRNA Isolation Kit (Roche) was used to cleanup samples with low RIN values. Additional measures of RNA

quality are presented for the Arctic fox study, since cross-species hybridization of cDNA microarrays requires stringent RNA quality standards (see Appendix A).

2.3.4.3 Targeted gene analysis by TaqMan based quantitative RT-PCR analysis

Genes selected for q-RT-PCR analysis (n=18) were chosen based on involvement with sulfur and selenium based antioxidant systems and are presented in Table A2.1. Synthesis of cDNA was done using SuperScript III Reverse Transcriptase and Random Hexamers according to the manufacturer's protocol. Primers utilized were selected based on canine primers available from Applied Biosystems (TaqMan Gene Expression Assays), and context sequences are available in Appendix A (see Table 2.A-1). q-RT-PCR was conducted using the Biomark HD System by Fluidigm (Fluidigm, San Francisco, CA) and Applied Biosystems Sequence Detection System (ABI 7900, Life Technologies, Grand Island, NY).

2.3.4.4 Microarray processing and data analysis

The Functional Genomics and Proteomics Facility Core of the Center for Ecogenetics and Environmental Health at the University of Washington followed the manufacturer's protocols for processing Affymetrix GeneChip® Canine Genome 2.0 Arrays (Santa Clara, CA). Hybridized arrays were scanned with an Affymetrix GeneChip® 3000 scanner. Raw microarray data were pre-processed and analyzed with Bioconductor (http://www.bioconductor.org/).[32] Several quality control steps were carried out to ensure data was of high quality: 1) visual inspection of the GCOS DAT chip images, 2) visual inspection of the chip pseudo-images generated by the Bioconductor affyPLM package, 3) generation of percent present calls and average background signals with the Bioconductor simpleaffy package, 4) generation and inspection histograms of raw signal intensities, and 5) generation and comparison of the Relative Log Expression and Normalized Unscaled Standard Errors using the Bioconductor affyPLM package. All microarray data derived from Affymetrix GeneChip® Canine Genome 2.0 arrays used in this study have been deposited in the Gene Expression Omnibus Database under accession number GSE71407 (http://www.ncbi.nlm.nih.gov/geo/).

2.3.4.5 Hg analysis

Total Hg was measured as in Lieske et al.[30] Briefly, total mercury concentrations ([THg]) were measured in whole blood from sled dogs and Arctic fox using a DMA-80 analyzer (Milestone, Sorisole, BG, Italy) in at least 2 repeated samples. Blanks were below 0.1ng THg, and standards including DORM-3 (National Research Council Canada), Lake Superior 1946 frozen fish homogenate (National Institute of Standards and Technology), IAEA-086 Human Hair (International Atomic Energy Agency Analytical Quality Control Services) were all within 10% of their expected values. For further details of THg analysis in canids see [13,30].

2.3.4.6 PCB analysis

PCB concentrations were determined in fox whole blood following modified procedures of Sloan et al. and Knott et al. [33,34] Individual PCB congeners were analyzed using low resolution quadruple gas chromatograph/mass spectroscopy (GC/MS) at the NOAA/NMFS Montlake laboratory in Seattle, WA. Each sample batch (n = 14) included a series of external standards and were based on a five-point calibration curve. Percent recoveries for these batches of samples were 98.7 ± 1.7%, 102.1 ± 1.9%, and 100.0 ± 15.9% for internal spike (PCB103) in blood/standard reference material (SRM)/method blank, certified congeners from SRM, and congeners of duplicates, respectively. All method blanks analyzed were below the limit of detection (no blank correction used).

Details regarding solvent extraction, filtration, cleanup, calibration, individual congeners analyzed, QA/QC is available in the Appendix A.

2.3.4.7 Statistical analyses

All statistics and figures were generated using R programming language and ggplot2 package [35,36] as well as the Bioconductor packages limma, qvalue, affy, and affyPLM. [37–40]

2.4 Results/Discussion

2.4.1 q-RT-PCR based gene expression analysis in fish fed sled dogs sampled over 13 weeks

We used q-RT-PCR to assess expression of genes associated with heavy metal metabolism and oxidative stress including CARM1, GCLC, GCLM, GPX1, GSTA3, GSTA4, GSTM3, GSTP1, GSTT1, MPST, MT1F, MT2A, MT4, MTR, SLC1A4, SLC1A5, SLC3A1, and TST (see Table 2.A-1). By examining the toxicodynamics of THg in WB (Figure 2.3), we concluded that the concentration of THg plateaued starting at approximately Week 11. Using a repeated-measures ANOVA with the error nested in individual variation per treatment group across all weeks of the treatment, no genes were determined to be expressed differently between treatment groups although the power of these models was hindered by large variability in individual gene expression levels and low sample size. By comparing the fold-change difference in expression from Week 0 expression to Week 11 using post-hoc paired t-tests, one gene (metallothionein 2A (*MT2A*)) was determined to be differentially expressed between the two treatment groups following Holm multiple test correction (Figure 2.4a, $p<0.01$). However, upon examination of individual dog expression profiles over the course of the feeding trial, it is clear that some animals displayed 2-10 fold up-regulation and down-regulation over the course of the experimental period (e.g. expression of MT2A shown in Figure 2.4b). If we simply assessed Week 11 alone, and not the time course variation in *MT2A* we could have erroneously concluded a biologically and statistically significant difference in expression. We illustrate here that evaluating sulfur-dependent antioxidant systems using a standard pre- versus post-diet analysis of gene expression does not adequately capture the variability and stochasticity of domestic animal models over the temporal scale utilized in this experiment.

While Lieske et al. [30] estimated dietary THg intake, the nutritional content and contaminant concentrations of fish are very different than kibble alone. Bouwens et al. demonstrated gene expression changes in response to dietary supplementation of fish oil in human peripheral blood monocytes (PBMCs).[27] Although the animals were of similar age, sex, and body condition, individual variation likely plays a large role in both baseline expressions of inducible genes as well as individual response to

environmental stimuli. [41] Indeed, our data seem to support both observations, because while the mean expressions values of some genes were consistently different between the two groups, the variability within groups (i.e. individual variation) as well as small sample size prevented differences in expression from being rigorously elucidated. We emphasize that we are not attempting to describe a dose-response relationship as a different study design would be needed. While it is conceivable that there may be changes in transcriptional profiles at low doses of mercury, Radonjic et al. [42] only found altered gene expression in cerebellums of developing rats at $MeHg^{+}$ doses that were one order of magnitude higher than the estimated dose in this study (0.100 mg/kg body weight, versus 0.013mg/kg body weight this study). The current study design featured continuous dietary exposure to Hg [THg], thus time (week) and [THg] in WB were highly correlated (r=0.91). Thus it would be not feasible to try and tease out the actual effects of Hg on gene expression using this study design as the use of fish as the vehicle for delivering Hg is not an inert method. It is interesting to consider that, while it would be difficult to assign changes in gene expression to Hg in fish rather than other nutrients/contaminants, it may be worthwhile to compare the results from a fish-based feeding study to laboratory experiments of Hg exposure. In other words, it is important to consider that changes in physiology associated with Hg exposure in a laboratory setting might be absent in a fish based diet exposure due to protective nutrients collocated in fish (i.e. selenium, polyunsaturated fatty acids). [43]

Many toxicological, pharmacological, and nutritional studies have been designed to describe changes in gene expression associated with a treatment, yet there is a paucity of data concerning "normal" or "baseline" expression levels to which to compare treatment groups (e.g. fish fed and no fish in diet groups). Whitney et al. found effects of sex, age, and time-dependence (what time of day blood was drawn) considered 2-fold differences in expression levels as over- or under-expressed.[41] While there have been a few studies that have examined environmental toxicant-induced changes in gene expression over small time scales (i.e. 8-12 hours, [29]), very few have examined the transcriptomic response of a model organism over extended time periods. In the present study, we found that some individuals, even those

maintained on the kibble diet, demonstrated 2-fold increases and decreases in gene expression on numerous time-points over the course of the study (see Figure 2.4). While it is conceivable that observed changes in gene expression could be related to other environmental factors that were not controlled for in the present study, we argue that establishing values for variation in baseline (i.e. normal) expression is critical for assessing canine toxicogenomics, and should be used to develop and validate potential biomarkers.

2.4.2 Mixtures of Hg and PCBs in Arctic foxes

A full description of methods for assessing RNA quality and probe hybridization in this cross-species assessment is presented in the Appendix A. The RNA quality from the 11 Arctic fox samples here fell within the benchmark levels used to assess performance on this array (Table 2.2).

The PCB and Hg concentrations measured are well within the range of concentrations previously reported and expected (see Table 2.2). Analysis of microarray gene expression resulted in relationships with sex, and concentrations of Hg or PCB for numerous genes known to be associated with these measures from previously published research, although small sample size and strict false-discovery control reduced the power of our models. For the model comparing gene expression by sex a significant difference was noted for eukaryotic translation initiation factor 2, subunit 3 gamma (*EIF2S3*) that has been documented to be more highly expressed in male dogs. [44] The expression in the Arctic fox model was at least 2-fold higher in males (adjusted p-value = 0.003). The significance of this finding is outlined by Nicolson et al. for the domestic dog, and would apply to other canine models in that gender dimorphic toxicity should be considered an important factor to address.[44] They investigated gene expression in the heart (ventricle and atrium), ileum, liver and kidney (medulla and cortex) where they showed that *EIF2S3* is consistently highlighted across all six tissues examined (nearly three times over-expressed in male dogs).[44] We document a similar finding in peripheral blood samples of the Arctic fox, and this provides another piece of evidence that this domestic dog microarray has utility for our model addressed here (conserved elements related to sex differences).

We found some evidence that thyroid hormone receptor interacting proteins (*TRIP6* and *TRIP10*) have an altered expression related to sex and concentrations of toxicants in Arctic foxes, although following p-value adjustment the results were not statistically significant. Gauger et al. indicated that PCBs may interfere with thyroid hormone (TH) signaling by reducing TH levels in blood, by exerting direct effects on TH receptors (TRs). [45] Miyazaki et al. showed hydroxylated-PCB at doses as low as 10^{-10} M suppresses TH-induced transcriptional activation of TR. [46] Similarly Kirkegaard et al. [18]found lower concentrations of T3 and T4 in plasma from sled dogs fed contaminated minke whale blubber compared to animals fed a control diet. In our models some evidence of interactions with PCBs was noted, but it was not conclusive. Although low sample size prevented robust analyses of mixture effects on gene expression, we note that in light of the Arctic foxes' diverse foraging ecologies and the ability to retrieve high-quality RNA from remote field sites, the Artic fox represents a unique model for understanding toxicity of contaminant mixtures in Arctic ecosystems that can be enhanced using domestic canine-based molecular tools.

2.4.3 Sled dogs and particulate matter pollution

Weldy et al. reported the glutathione (GSH) synthesis genes (*GCLC* and *GCLM)* were induced by exposure of endothelial cells and macrophages to collected diesel particulates *in vitro*. [47] In our study, there was no difference in *GCLC* expression between the two exposure groups, although this may reflect exposure route of the particulates between our study and Weldy et al. (*in vitro* cell culture versus respiratory exposure and circulating leukocytes).[47] No differences were observed in clinical examination variables (i.e. weight, temperature) or hematological variables (i.e. complete blood count, full list presented in Montrose et al. [31]) between the two exposure groups. While mean values were often unequal, large intra-exposure group variability prevented comparisons from being statistically significant. Similarly, gene expression did not vary between exposure levels, indicating that if there is indeed a measurable physiologic or transcriptomic response to observed levels of $PM_{2.5}$ exposure, it could not be detected using these metrics or analytics.

The pathway of $PM_{2.5}$ injury remains unclear, and it is important to identify potential confounding variables associated with gene expression levels if transcriptomic approaches are to be effective. Because of the relatively large sample size (n=28-30 animals per kennel) and the exploratory nature of this study, we investigated potential confounding variables related to gene expression and physical and hematological variables.

$PM_{2.5}$ has been observed to induce oxidative stress in peripheral blood. [48] Glutathione peroxidase (GPx) activity is a common biomarker of oxidative stress. In this study, we did not detect any link between $PM_{2.5}$ "dose" and *GPX1* (coding for glutathione peroxidase (GPx)-1 isozyme) expression, although we did note a significant relationship between *GPX1* expression and canine body mass (Figure 5a n=70, min= 17.7kg, max=32.2kg, median=23.8kg, $p<0.01$, $r=0.30$). GPx activity has previously been associated with measures of body condition. Espinoza et al. found that body mass index (BMI) was significantly positively associated with *Gpx-1* expression at <30 kg/m^2, and Ozata et al. found significantly greater GPx levels in obese individuals (BMI > 39kg/m^2) versus "healthy" individuals (BMI 21.75±1.87 kg/m^2).[49,50] None of our study animals were determined to be obese, no doubt due largely to the incredible fitness of Alaskan sled dogs, however we did note that heavier animals tended to have higher levels of *GPX1* expression compared to smaller animals.

Another biomarker of oxidative stress, 4-hydroxynonenal (4-HNE) is produced as a by-product of lipid peroxidation and is biotransformed via GSH-dependent detoxification systems. *GSTA4*, coding for glutathione-s-transferase (*GST*) isozyme 4, has been shown to have catalytic efficiency toward conjugating 4-HNE to GSH, and thus is an important enzyme in the 4-HNE induced apoptotic pathway. [51] We found that expression of *GSTA4* in dogs was significantly affected by the proportion of specific leukocyte populations (in this case percent granulocytes, %GRA) in the blood. Similarly, *GSTP1 (*GST-pi 1), which has been shown to be induced by oxidative stress in cultured human cells, showed a significant positive relationship with lymphocyte percentage (Figure 5b). [52] Gene expression profiles have been shown to differ across leukocyte populations in humans, and it could explain the observations

presented here.[53] An alternative explanation is that both gene expression and leukocyte profiles are co-varying with another physiological variable that was not recorded in this study. Regardless, we emphasize the benefit of accompanying hematological data with assessments of gene expression in PBMCs in canid models.

The biological significance of these correlative findings is uncertain, but does serve as a cautionary caveat (e.g. confounding variables) to interpreting transcriptomic data especially in natural systems (i.e. uncontrolled settings). In toxicogenomics studies where the pathway of cellular injury is not well described (i.e. PM exposure), it may be highly valuable to collect hematological and physical examination data on the study animals.

The use of Arctic canid species as models for investigating molecular toxicology of environmental contaminants presents a number of intriguing possibilities. Wild canids represent a number of ecosystems and feeding ecologies, while large kennels of domestic dogs (sled dogs) share similar diet and air pollution exposure to humans.[17] While we note that our initial studies revealed few significant differences in gene expression between exposure groups, we demonstrated that (1) successful hybridization of Arctic fox cDNA to a domestic dog array support the use of Arctic fox in molecular toxicology and (2) sled dogs can be used as environmental sentinels to monitor changes in gene expression brought on by varying exposure to contaminants via diet or air pollution. Although we interpret our initial correlative findings with caution, we feel that, with improved study design and control, Arctic canid models have the potential to elucidate changes, if any, in gene responses to environmental contaminants. The results of these findings would have implications for not only describing biological responses to contaminants, but the health and status of Arctic wildlife populations and human inhabitants.

We have shown here that collecting gene expression data, in particular data collected from PBMCs, is feasible for field-based studies of free ranging and domestic populations. Using stabilization

techniques, high quality total RNA can be extracted from whole blood samples collected in remote field conditions, widening the scope of molecular toxicology investigations. While PBMCs represent an interesting tissue to examine for gene expression and can elucidate questions involving potential immunotoxicity and immune function, there are a number of questions that remain concerning baseline variability in gene expression and temporal response of gene transcription to toxic insult. Future investigations should establish methods for assessing sources of variation and stochasticity in PBMC gene expression in order to advance the field of molecular toxicology in wildlife and domestic animal populations.

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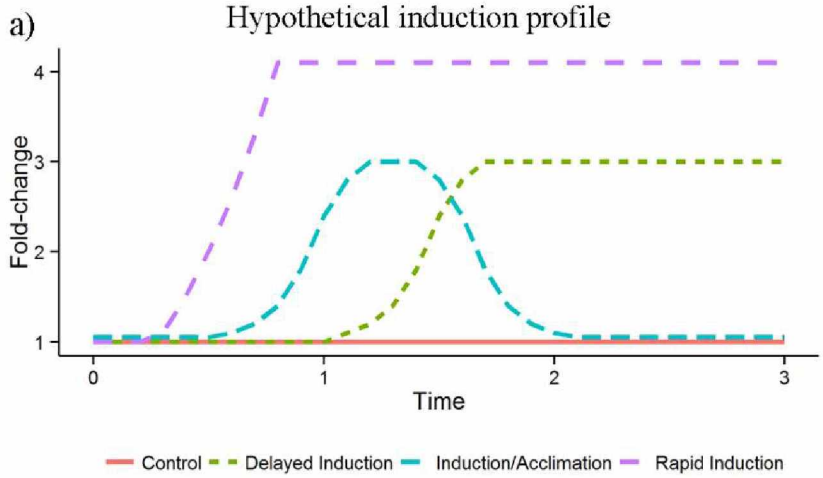
2.6 Figures

Figure 2.1 - The boundary of the Arctic Monitoring and Assessment Program (AMAP) that will be considered as the delineation for the "Arctic" in this study. Figure from AMAP (2009).

a)



b)

Sampling Time	Gene Response		
	Delayed Response	Response/Acclimation	Rapid Response
0	Baseline	Baseline	Baseline
1	No response	2-fold induction	4-fold induction
2	3-fold in0064uction	No response	4-fold induction
3	3-Fold induction	No response	4-fold induction

Figure 2.2 - Hypothetical up-regulation (a) and down-regulation (b) profiles for genes expressed over time in response to environmental contaminants. By sampling at time 0 (baseline) and comparing the expression of these genes to post-treatment time points (time 1-3) it is clear that some gene response/time points will result in variable ability to detect biological response.

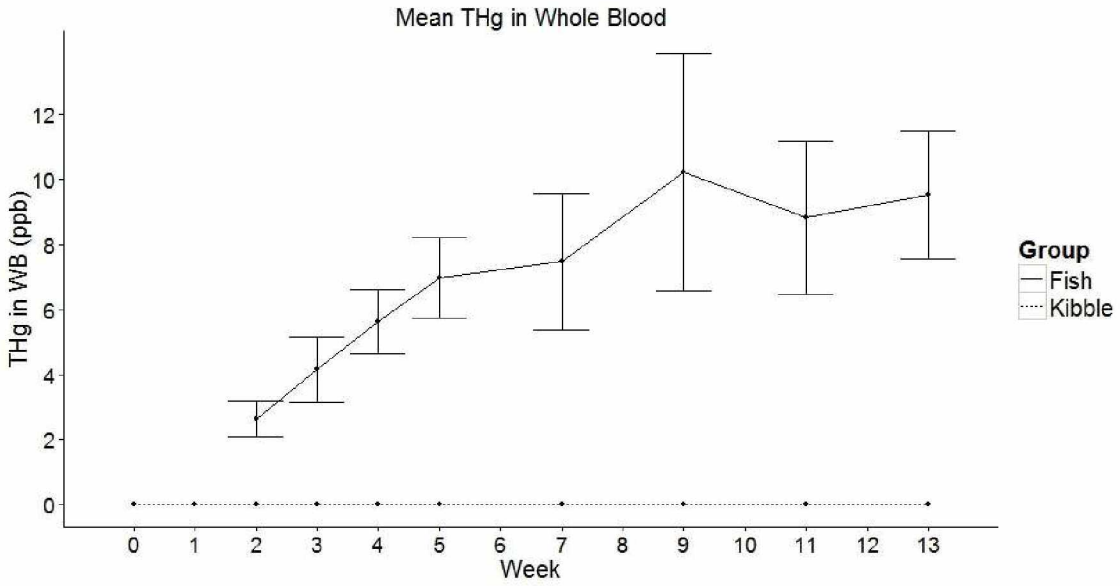


Figure 2.3 - Concentrations of THg in WB measured in sled dogs fed 50% fish and 50% kibble (Fish group, n=4) and 100% kibble (kibble group, n=4). THg values for the kibble group were below detection limit for all weeks sampled. Data from Lieske et al. [30]

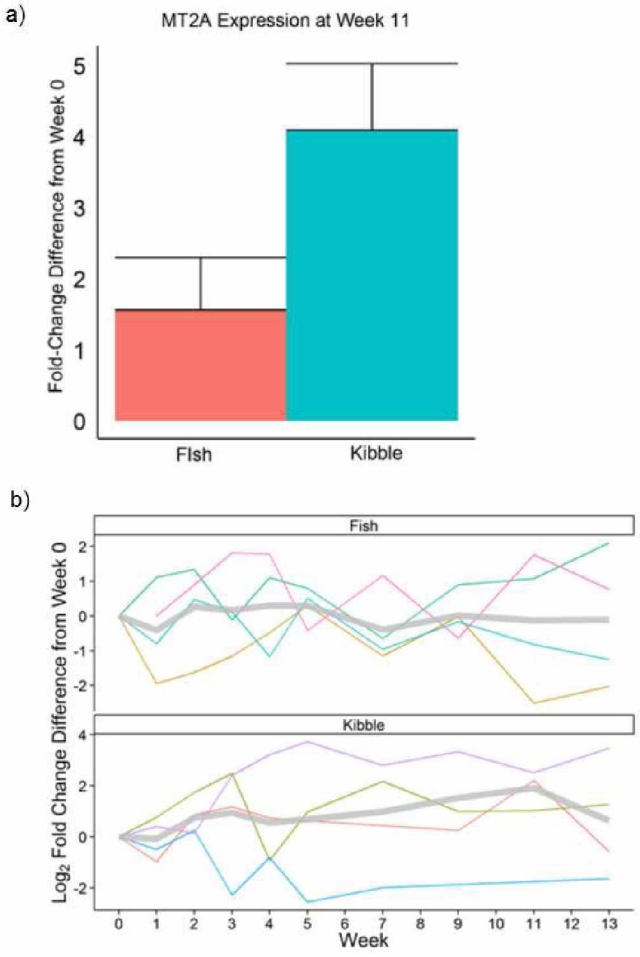


Figure 2.4 - (a) *MT2A* expression at Week 11 (fold change from Week 0) was determined to be significantly different between treatment groups. Error bars represent standard error (b) However some dogs showed large variability in *MT2A* expression (log_2 fold-change) for Week *n* compared to Week 0 over the course of the feeding trial. Here the grey lines represent the mean for each treatment group per week. Each colored line represents an individual animal. We emphasize that single-time point analysis of gene response might not reflect variability found in *in vivo* studies.

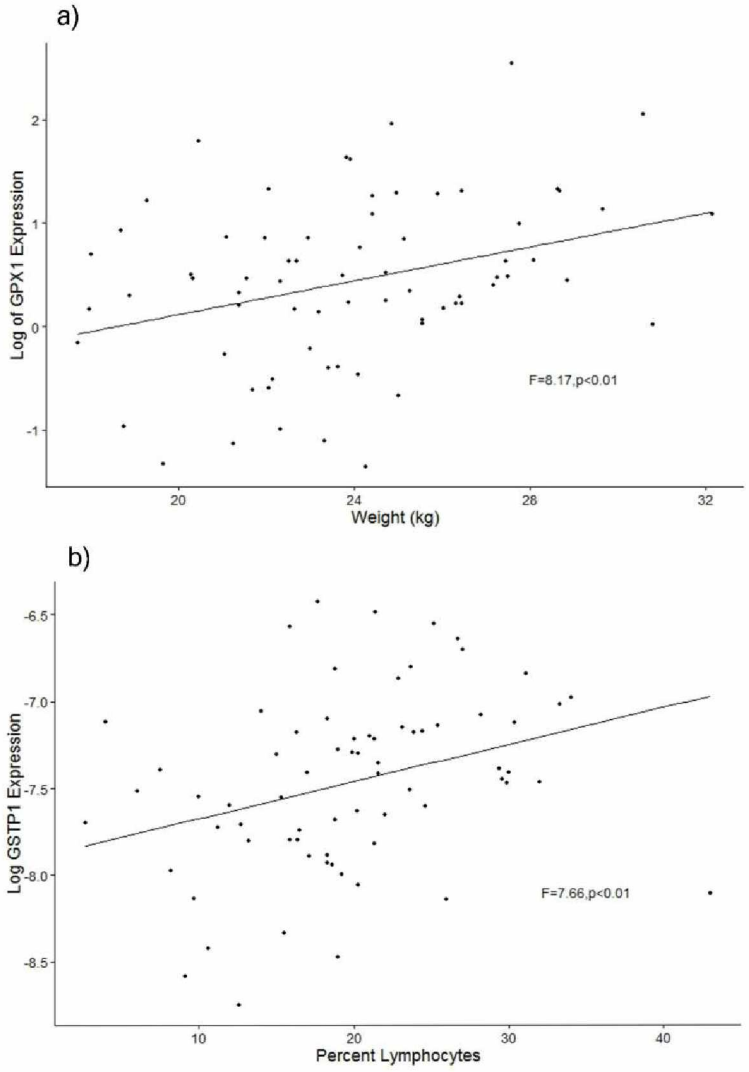


Figure 2.5 - (a) Log_2 expression of GSTP1 was positively associated with the percent lymphocytes in the whole blood sample and (b) log_2 *GPX1* expression was positively correlated with weight of the animal (kg) ($p<0.01$).

Table 2.1 - [THg] in hair and blood and [∑PCBS] in blood for 11 Arctic foxes caught in 2007. Blood [THg] values are shown as wet weight in parts per billion, hair [THg] values are given as dry weight in parts per million. [∑PCBS] values in blood are shown for 40 PCB congeners and expressed in (ng/g) wet weight.

Animal ID	Sex	Blood THg (ppb)	Hair THg (ppm)	Blood ∑(40)PCBs (ppb)
AFX07-001	M	12.3	0.55	2.47
-002	F	20.6	0.81	2.97
-003	M	17.9	1.02	0.18
-004	M	5.5	0.39	0
-005	F	27.9	1.46	0
-006	M	22.1	0.59	0.51
-007	F	12.2	0.44	0
-008	F	26.6	0.74	0.77
-009	F	55.5	5.1	3.17
-010	M	18.3	0.75	0.07
-011	F	31.4	1.73	4.67

2.7 Tables

Table 2.2 RIN values greater than the The Microarray Quality Control Consortium RNA Integrity Number (RIN) > 8.0 are highlighted in bold text.

		UAF Assessment		UW CEEH Assessment	
Sample ID	Animal ID	RNA yield	RIN	RNA yield	RIN
121	AF008	74.3	**8.1**	63	**8.8**
122	AF002	255.7	6.1	215	**8.9**
123	AF010	81.7	7.5	72	**8.6**
124	AF011	89.0	**8.6**	79	**9.2**
125	AF005	155.8	**8.1**	134	**8.7**
132	AF006	113.0	6.7	96	7.9
133	AF004	173.6	**8.3**	158	**8.9**
134	AF001	100.4	**8.2**	95	**8.8**
143	AF007	54.0	**8.2**	49	**8.4**
144	AF003	74.4	**8.6**	66	**8.4**
1B	AF009	33.2	7.8	33	7.7
	Mean	109.6	7.8	96.4	**8.6**
	SD	63.47	0.79	53.37	0.45
	Range	33.2 - 255.7	6.1 - **8.6**	33.0 - 215.0	7.7 - **9.2**

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2.9 Appendix A

2.9.1 PCB analysis

Polychlorinated biphenyl (PCB) concentrations were determined in fox whole blood following modified procedures of Sloan et al. and Knott et al.[1,2] Briefly, whole blood was mixed with sodium sulfate and magnesium sulfate (drying agents) in a 1.5 to 1 volume/volume ratio. Extraction was done using dicholormethane on an acceleratred solvent extractor (ASE 200, Dionex Corp., Sunnyvale CA). Extracts were filtered using gravity flow silica gel columns, and subsequently run through size exclusion high performance liquid chromatography (SEC-HPLC). PCB congeners were at the National Oceanographic and Atmospheric Administration (NOAA)/National Marine Fisheries Service (NMFS) Montlake Laboratory in Seattle, WA using quadruple gas chromatograph/mass spectrometer (GC/MS). Method detection limits ranged from 0.07 - 0.79 ng / g (lowest of calibration range). Concentrations were reported as sum of 40 PCB congeners (∑40 PCB; 17, 18, 28, 31, 33, 44, 49, 52, 66, 70, 74, 82, 87, 95, 99, 101/90, 105, 110, 118, 128, 138/163/164, 149, 151, 153/132, 156, 158, 170/190, 171, 177, 180, 183, 187, 191, 194, 195, 199, 205, 206, 208, 209). PCB congeners 99, 105, 138, 153, 170, 180, and 194 (∑PCB7) were used in statistical analyses. Each sample batch (n = 14) included a series of external standards and were based on a five-point calibration curve. Percent recoveries for these batches of samples were 98.7 ± 1.7%, 102.1 ± 1.9%, and 100.0 ± 15.9% for internal spike (PCB103) in blood/SRM/method blank, certified congeners from SRM, and congeners of duplicates, respectively. All method blanks analyzed were below the limit of detection (no blank correction used).

2.9.2 Arctic fox/ domestic dog homogeneity assessment

The mean RNA quantities were 109.6 (UAF) and 96.4 (UW) ng/µl for the 11 Arctic fox samples (Table 2.2). In order to assess RNA quality, the RNA Integrity Number (RIN) for each sample was determined, which uses a Baysian framework to assess and standardize electrophoregraph RNA measurements.[3] For the UAF analysis RIN was >8.0 for 7 of the 11 samples and 9 of the 11 samples

(post-shipping) for the UW analysis; with a mean RIN of 7.8 (pre-shipping, UAF) and 8.6 (post-shipping, UW) (see Table 2.2).

As an additional assessment of RNA quality we considered two sources of "noise" as previously described by Popova et al. [4] as 1) random factors via the technology (e.g. low abundance of transcripts, platform, sample handling, etc.) and 2) more systematic factors related to the nature of the transcripts and the probes (e.g. probe set distribution, length and stability of mRNA species). In the study by Popova et al. [4] the post chip housekeeping genes for the Affymetrix UG133 chip (β-actin and GAPDH) were used by comparing the 3'/5' ratio. They stated that this index assesses the original integrity of the RNA, accuracy of sample processing such as RNA purification, reverse transcription, *in vitro* amplification and labeling, fragmentation and hybridization. A value of <3.0 for the 3'/5' is considered to represent high quality RNA; while >3.0 may indicate low quality material and/or sample processing issues. Poor RNA quality could lead to spurious results, especially with lack of species specific arrays, new sample matrices and non-target species (e.g. use of samples from closely related species from which the array was derived; in this study fox samples were used on a domestic dog derived array). We conclude we have very good RNA integrity for these Arctic fox samples and that these domestic dog-based arrays are performing adequately for the Arctic fox.

A confounding factor in the use of microarrays in cross-species assessments is the effect of sequence divergence between distinct lineages. [5] Gilad et al. [6] found that there was a significant effect of sequence mismatch on probe hybridization when comparing different primates (humans, chimpanzee, orangutan, and rhesus monkey). When sequences diverged by 1% (human compared to chimpanzee) they found that 63% of probe hybridizations were significantly different due to sequence mismatch, and in 5% sequence divergence (human compared to rhesus) they found that 89% of probe hybridizations were affected. However, we emphasize that we are not attempting to compare expression levels between species, (i.e. comparing Arctic fox versus domestic dog), and while intraspecies comparisons may still be subject to sequence mismatch (i.e. natural populations may have >1% genetic diversity) Gilad et al. [6]

concluded that cross species comparisons may still be effectively used to identify genes that are highly differentially expressed (greater than 1.5 fold change in expression). Thus, using a similar fold-cutoff value in our study is warranted. The sequences of the artic fox genes were determined for each PCR amplicon derived from Arctic fox cDNA. Seven Arctic fox genes were aligned to reference sequences available for the domestic dog using the Basic Local Alignment Search Tool (BLAST). [7] The genomic sequence alignments for domestic dog genes *SAPS3*, *GAPDH*, *ZCCHC11*, *RYK*, and the corresponding artic fox genes showed 93.3%, 97.6%, 99%, and 99% homology, respectively.

2.9.3 Microarray performance

Numerous Affymetrix quality assurance measures revealed that the arrays used in this study performed at manufacturer's published standards, indicating that the sample collection protocols used in remote field efforts were able to maintain adequate quality samples.

Relative log expression values (RLE) values are computed for each probeset by comparing the expression value on each array against the median expression value for that probeset across all arrays. Assuming that most genes are not changing in expression across arrays means ideally most of these RLE values will be near 0. Boxplots of these values, for each array, provides a quality assessment tool. Typically arrays with poorer quality show up with boxes that are not centered about 0 and/or are more spread out. In Figure S2.1, all 11 samples closely centered about zero.

For Normalized Unscaled Standard Errors (NUSE), the standard error estimates obtained for each gene on each array are taken and standardized so that the median standard error for genes is 1 across all arrays. This process accounts for differences in variability between genes. An array where there are elevated SE relative to the other arrays is typically of lower quality. Good values should be between 0.95 and 1.05. In Figure S2.2, medians of all 11 samples between 0.95 and 1.05, indicating good values.

2.9.4 Figures

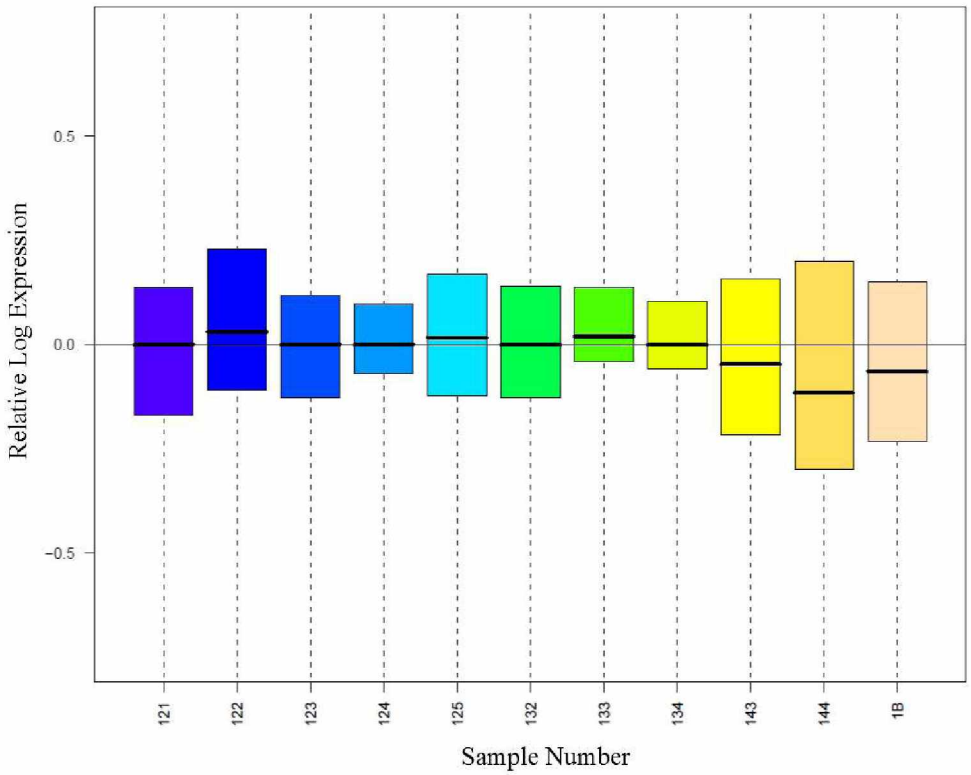

Figure 2.A-1 Relative Log Expression (RLE) values showing good array quality.

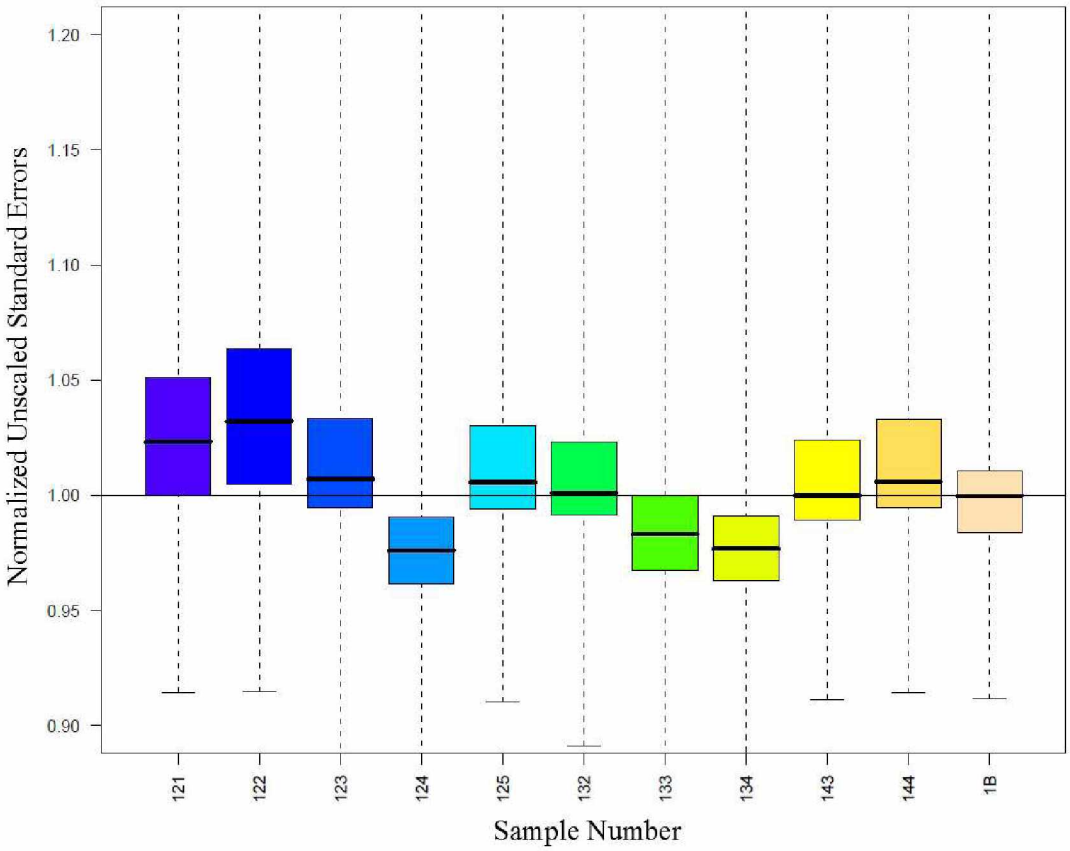

Figure 2.A-2. Normalized Unscaled Standard Errors (NUSE) showing good agreement between arrays.

2.9.5 Tables

Table 2.A-1 Genes targeted for expression analysis during the controlled feeding study. Gene IDs and descriptions from NCBI (http://www.ncbi.nlm.nih.gov/).

Gene Symbol[1]	ENTREZ Gene ID	Protein	Assay ID[2]	Context Sequence
CARM1	484947	Coactivator-associated arginine methyltransferase 1	Cf02731369_m1	GGGATTGTCCAAGGGTCCTCCGGCG
GCLC	609822	Glutamate cysteine ligase catalytic subunit	Cf02638863_m1	AGAGAGCATCTGGCGAACTAATGAC
GCLM	612283	Glutamate cysteine ligase regulatory subunit	Cf02660895_m1	ATTTGGTCAGGGAATTTCCAGATGT
GPX1	442961	Glutathione peroxidase 1	Cf02625205_g1	CAGTTCGGGCATCAGGAAAACGCTA
GSTA3	474938	Glutathione S-transferase alpha3	Cf02625274_m1	TGTTTGAAAAAGTGTTAAAGAGCCA
GSTA4	474939	Glutathione S-transferase alpha 4	Cf02662188_m1	CGTCTGCTACAGGCATTTAAAACAA
GSTM3	479911	Glutathione S-transferase alpha 3	Cf02638034_m1	AGGAGAAAAGCTCACCTTTGTGGAT
GSTP1	476005	Gutathione S-transferase pi 1	Cf02642382_m1	CCACCACAGCTATGAGGGAGGCAAG
GSTT1	477556	Glutathione-s-transferase theta 1	Cf02633963_m1	GTGGCATAAGGTGATGTTCCCTGTT
MPST	474515	Mercaptopyruvate sulfurtransferase	Cf02694844_g1	TCGCCCTACGACCACATGCTGCCCA
MT1F	403800	Metallothionein 1F	Cf03417985_s1	TTTTTCATATCACTCTGACTTGTTT
MT2A	403768	Metallothionein 2A	Cf02622008_gH	GCGCCGCGGGGGGCTCCTGCACGTG
MT4	403769	Metallothionein 4	Cf02622007_m1	CATGTCGAAAAAGCTGCTGTCCTTG
MTR	479190	5-methyltetrahydrofolate-homocysteine methyltransferase	Cf02690558_m1	CTCGTCATGGCAGGAAACTATGAAG
SLC1A4	481391	Neutral amino acid transporter A	Cf02649495_m1	GTGGACTGGATTGTGGACCGCACCA
SLC1A5	484425	Neutral amino acid transporter B	Cf02730483_g1	TGTGAAGATCATCACCATCCTGGTC
SLC3A1	403700	Neutral and basic amino acid transport protein rBAT	Cf02623862_m1	TTCAATGAAAGCTATGATGTTAATA
TST	481275	Thiosulfate sulfurtransferase	Cf02648215_m1	CAGATGCAGTAGGACTGGACTCCGG

[1] Documentation for GAPDH (assay ID AIKAKMS) provided primer sequences rather than context sequence (forward=CCCCAATGTATCAGTTGTGGATCTG; reverse=CCTGCTTCACTACCTTCTTGATGTC).

[2] Assays were purchased through Applied Biosystems, Foster City, CA,

Table 2.A-2 A description of particulate matter (PM) fractions and selected health effects.

Description of PM Fraction	Particle Size	Health Effects
"Coarse"	2.5μm - 10μm	Cytokine induction and cytotoxicity (Monn & Becker, 1998)
"Fine"	<2.5μm	Potential trigger for myocardial infarction (Peters et al., 2001);
"Ultra-Fine"	<0.1μm	Oxidative stress and mitochondrial damage (Li et al., 2003)

2.9.6 Appendix A Works Cited

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Chapter 3 –**Novel applications of next generation sequencing tools to assess the health of Steller sea lion (Eumetopias jubatus) populations**[3]

[3]Harley, J. R., J. M. MacDonald, L. D. Rea, T. K. Bammler, T. J. Kavanagh, B. S. Fadely., T. Gelatt., and T. M. O'Hara. In Revision. Novel applications of next generation sequencing tools to assess the health of Steller sea lion (*Eumetopias jubatus*) populations. Science of the Total Environment.

3.1 Abstract

Some rookeries of the western distinct population segment (WDPS) of Steller sea lions (*Eumetopias jubatus*) in the Aleutian Islands (Alaska, USA) have experienced continued declines since the initial collapse of the population in the 1970-1980s. Several theories have been put forward to explain the decline and lack of subsequent recovery including predation, nutritional stress, contaminants, and infectious disease agents, but thus far a primary cause has not been identified. Examining gene expression profiles of organisms has been promoted as a way to assess several health indicators related to toxicoses, infection, and nutritional stress using recent advances in metagenetics (next-generation sequencing) analyses. Next-generation sequencing may provide a more refined and adaptable method of investigating sea lion health under difficult research field collections. Here we suggest that the application of next-generation sequencing tools has the potential to evaluate the transcriptomic (gene expression) profile of animals from declining rookeries. We show that high quality RNA can be obtained from wildlife populations despite logistically challenging field conditions. We compared RNA expression in whole blood using whole transcriptome sequencing (RNA-Seq) among animals with relatively high concentrations of total mercury ([THg]) to animals with lower concentrations. There did not appear to be significant changes in gene expression in animals with high [THg] in whole blood, despite some animals having concentrations above thresholds of concern for model organisms. We did find evidence of a bias toward downregulation of some genes in animals with higher [THg]. We used sequence data generated from RNA-Seq experiments to mine presence of potential pathogens (both bacterial and viral) using ContextMap, which identified sequences aligning to a haemotrophic *Mycoplasma spp.* in a blood sample. Despite being focused on molecular components of individual organisms, we argue that with further development and increased genomic information the tools and methods presented here have the potential to help elucidate responses to stressors and to the presence of potential disease-causing agents in endangered populations.

3.2 Introduction

The western distinct population segment (WDPS) of Steller sea lions (*Eumetopias jubatus*) experienced a sharp decline in the 1970 - 1990s from which the WDPS has not recovered (Trites et al. 2007, Allen and Angliss 2014). The WDPS decreased from an estimated >200,000 animals in the early 1970s (Trites and Larkin 1996) to a minimum of less than 50,000 individuals counted in 2000 (Atkinson et al. 2008, Allen and Angliss 2014). There is evidence that pup and non-pup counts in some regions of the WDPS have been increasing from 2003 to 2014, however some rookeries in the Aleutian Islands continue to show lack of recovery or decline (Fritz et al. 2013, Johnson and Fritz 2014).

The causes of the initial decline and the lack of subsequent recovery for rookeries of the WDPS have been vigorously debated and include theories related to prey availability, fisheries interactions, "junk food", nutritional stress, regional abiotic regime shifts, contaminants and infectious disease, and increased predation from killer whales (sequential megafauna collapse) (Rosen and Trites 2000, Springer et al. 2003, Dillingham et al. 2006, Trites et al. 2007). Most of these theories boil down to either top-down or bottom-up forcing, although it has been suggested that these disparate hypotheses could be coalesced using a holistic oceanographic framework (Trites et al. 2007). While there is evidence for large fluxes in historic population numbers based on archaeological and historical ecology data (Trites et al. 2007), there are a number of novel or more recent anthropogenic factors that may be limiting or altogether preventing the recovery of rookeries in the WDPS (Atkinson et al. 2008). It is known that some marine mammal populations are exposed to high levels of environmental contaminants relative to other mammals due to (1) high trophic level foraging ecology (piscivory), (2) long life span (many decades), (3) regional contamination (anthropogenic and natural), and (4) anatomy and physiology which supports sequestration of lipophilic contaminants in lipid rich tissues and other contaminants in other tissues (*e.g.* Bossart, 2011). Relatively high concentrations of contaminants, such as heavy metals, have been noted in numerous marine mammal species (Das et al. 2003), although it is often unclear what, if any, effects these contaminants have on the health of the individual and population when assessing free ranging marine

mammals (Beckmen et al. 2003, Ross 2004). Concentrations of mercury (Hg) for instance, exceed 100 μg/g total mercury (THg) in hair of harbor seals (*Phoca vitulina*) in a highly contaminated estuary (McHuron et al. 2014). The organic form of Hg, monomethylmercury ($MeHg^+$), bioaccumulates in marine organisms and is a well-known neurotoxicant, especially for the central nervous system (CNS) of the developing fetus and neonate (Morel et al. 1998). Due in part to the lack of defined neurological benchmarks or challenges associated with field sampling, examples of outright $MeHg^+$ toxicity in pinnipeds are rare. However, van Hoomison et al. (2015) found associations between abnormal neurological symptoms and increasing blood total mercury concentrations ([THg]) in harbor seals.

While the neurotoxic properties of $MeHg^+$ have been known for some time, the mechanism of toxicity is still being described and recent work has provided evidence that some Hg species have cytotoxic and immunotoxic effects as well. There are multiple lines of evidence to suggest that oxidative stress has a role in $MeHg^+$ induced neurotoxicity, especially during development (Johansson et al. 2007, Farina et al. 2011). $MeHg^+$ induces the formation of reactive oxygen species (ROS) in mitochondria which may leak out of the mitochondrial membrane and induce endoplasmic reticulum (ER) stress (Belletti et al. 2002, Usuki et al. 2008). Usuki et al. (2008) reported that $MeHg^+$ induced ER stress via ROS generation resulted in apoptosis in susceptible myocyte cell lines. Similarly, mitochondrial dysfunction has been implicated in observed immunotoxicity of $MeHg^+$ (such as T-cell apoptosis) (Shenker et al. 1998). The developing immune system appears to be additionally susceptible to the immunotoxic effects of $MeHg^+$, further increasing the value of examining neonates as a cohort of concern (Tonk et al. 2010). The use of circulating leukocytes makes it possible to examine gene expression changes in immune cells which are critical for health and development.

Large scale changes in gene expression following Hg^{2+} exposure have been demonstrated under *in vitro* and *in vivo* laboratory conditions (Ayensu and Tchounwou 2006, Ung et al. 2010). This sort of profiling is necessary for providing mechanistic descriptions of Hg^{2+} toxicity pathways, however exposure to solely Hg^{2+} (or $MeHg^+$) is an inappropriate context for examining potential health effects to

fish-eating mammals, where the addition of other nutrients (*e.g.* selenium (Se), sulfhydryl compounds (R-SH), polyunsaturated fatty acids (PUFAs)) could interact directly or indirectly with forms of Hg present in fish. For instance, Jayashankar et al. (2011) found that different sets of genes were regulated by Se, $MeHg^{+}$, and concurrent $MeHg^{+}$-Se exposure (at environmentally relevant concentrations) in cerebral tissue of mice. This suggests that examining changes in gene expression in wildlife could yield different results than exposure studies in controlled environments using different Hg exposure scenarios.

Nevertheless, profiling of global gene expression has been advocated as a relatively non-targeted method for assessing response to environmental contaminants (Wang et al. 2005, Thomas et al. 2014). The discovery of biomarkers associated with environmental exposures has contributed to the description of mechanistic pathways as assessments of physiological and pathological responses to toxicants (Forrest et al. 2005, Chaussabel 2015). Initial development of this technique relied heavily on the DNA microarray, however the continued development of next generation sequencing (NGS) techniques has left the microarray largely obsolete (Shendure 2008). While there are a number of bioinformatic hurdles associated with comparing gene expression from different NGS data sets, this technique has several advantages over microarrays. Firstly, reliable sequence data are archive-able for reanalysis in future projects. Secondly, sequence data can be uploaded to publicly available databases in order to further genetics research for non-model organisms; such as the Steller sea lion. And finally, the sequence data generated can potentially be mined for sequences which belong to organisms or agents other than the host, possibly representing microbial agents of interest (*e.g.* suspected pathogens, zoonotic agents, Bonfert et al. 2013).

The concept of metagenomics was first popularized in 1998 and described the identification of multiple species from environmental samples (Handelsman et al. 1998). Current metagenomics techniques rely heavily on NGS data and complex algorithms designed to assign sequences to different species (Tringe et al. 2005). While these methods were originally developed to describe populations of species in an environmental sample (*i.e.* soil), recent work has adapted these algorithms to search for

microbial sequences in the midst of a population of host sequences. This technique has been used successfully to identify viral and bacterial RNA sequences from a sample of human biological tissue (Bonfert et al. 2013). Furthermore, changes in host gene expression can be associated with evidence of pathogenic microbes, thus, as mentioned above, expanding the scope of the health and disease assessment beyond simply the host transcriptome.

Here we demonstrate several approaches for assessing the general health and disease status of a wildlife population, Steller sea lions, by using RNA sequencing techniques. We show that (1) high quality RNA-based samples can be obtained from remote field sites despite difficult logistical conditions, (2) RNA-Seq (whole transcriptome sequencing) can be used to investigate differences in gene expression related to concentrations of contaminants in blood, (3) and that RNA-Seq data can be mined using metagenomics tools to assess microbial agents of interest or concern.

3.3 Methods

Steller sea lion pups were captured[4] on 2 rookery islands in the WDPS (Agattu Island/Gillon Point, and Ulak Island/Hasgox Point) during June 24 - July 17, 2013 and June 18 - July 5, 2015 (Figure 3.1a). Each pup was manually restrained on a sampling board to administer isoflurane gas at 4 L/min O_2 and 4-5% vaporizer setting via a mask (Heath et al. 1997). The vaporizer was turned down to 2-3% after induction, with 2-3 L/min O_2 flow to maintain anesthesia. Blood samples were then collected via the caudal gluteal vein for THg analysis (Vacuette Trace Element, Geiner Labortechnik, Kremsmünter, Austria) and for RNA stabilization and collection (PaxGene™ RNA, PreAnalytiX, Hombrechtikon Switzerland). Blood was stored at approximately -15°C for the duration of the cruise (approximately 3 weeks), and placed in a -80°C freezer upon arrival at UAF.

[4] Permit Information: Alaska Department of Fish and Game; Marine Mammal Protection Act (MMPA)/Endangered Species Act (ESA) Permit 14325, Marine Mammal Laboratory MMPA/ESA permits 14326 and 18528, University of Alaska Fairbanks (UAF) Institutional Animal Care and Use Committee (IACUC) protocol #594759-2, Alaska Fisheries Science Center (AFSC)/Northwest Fisheries Science Center (NWFSC) IACUC assurances 2010-4 and 2013-2)

3.3.1 THg analysis

Whole blood (WB) [THg] were analyzed by combustion and atomic absorption using a Direct Mercury Analyzer (DMA-80, Milestone) in a similar manner to Castellini et al. (2012) and Rea et al. (2013). Mercury in 3.7% HCl, DORM-4 (Certified Reference Material, National Resource Council, Canada) and Seronorm (SERO, Billingstad, Norway) were used as standard reference materials. All samples were run in duplicate, except in cases where percent relative standard deviation (%RSD) between the two samples was greater than 10%, in which case samples were run in quadruplicate or higher. Runs were averaged to provide a single value for each sample. Recovery of standard reference materials averaged (±sd) 92.1±2.6% (Hg in 3.7% HCl), 100.4±5.0% (DORM-4, NRC) and 86.4±0.4% (Seronorm).

3.3.2 RNA-Seq samples

RNA-Seq samples were selected from Agattu (n=18) and Ulak (n=6) Islands, both of which have declining pup counts from 2000 to present (Fritz et al. 2015). In order to maximize study efficacy while working with limited funding, we decided to analyze samples with high and low [THg] (extremes) rather than treating [THg] as a continuous variable. We selected six individuals with the highest [THg] and lowest [THg] from each year of sampling (2013 and 2015). We initially included individuals from Ulak in 2013 to attempt to reduce the effect of differences between rookeries (see Figure 3.2).

3.3.3 Body condition scores

Body condition indices (CI, unitless) were calculated as a ratio of the axillary girth to the dorsal standard length as in (Hastings et al. 2011, Rea et al. 2016) using:

Equation 3.1

$$CI = \frac{axillary\ girth\ (cm)}{standard\ length(cm)} \times 100$$

By examining the gross morphometrics of our study individuals we could potentially exclude animals with extremely poor body conditions (*i.e.* due to poor maternal investment) which would likely influence global gene expression). We also performed a robust linear regression (minimizing the influence of outlying values) in order to determine the correlation between [THg] and CI.

3.3.4 RNA extraction and QA/QC

Whole blood collected in PaxGene™ RNA tubes were placed in a cooler with ice until transport back to the ship, at which point they were kept in the freezer at approximately -15°C. Upon return to UAF the samples were placed in a -80°C freezer and then shipped to the University of Washington Center for Exposures, Diseases, Genomics and Environment in insulated shipping containers with dry ice and subsequently stored at -80°C until extraction. Total RNA was extracted using the PaxGene™ Blood RNA Kit according to the manufacturer's protocol. RNA integrity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California). RNA quantity was determined by measuring OD_{260} with a Thermo Scientific NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Inc.; Wilmington, DE). The NanoDrop instrument was also used to determine purity of RNA samples by measuring $OD_{260/280}$ and $OD_{260/230}$ ratios. Only samples with RNA integrity numbers (RIN) > 8 (Table 3.1), and $OD_{260/280}$ and $OD_{260/230}$ ratios >1.8 were used for RNA-Seq analysis.

3.3.5 Transcriptome assembly

We used the 2015 data (including the three females) to generate a *de novo* transcriptome using the Trinity aligner, version 2.1.1 (Grabherr et al. 2011). We generated approximately 624K contigs, with a mean length of just over 1 kb; the smallest contig was 201 bp in length, and the largest was 26 kb in length. To assess quality of the transcriptome we used transrate (Smith-Unna et al. 2016), which reported an assembly score of 0.71 (optimal score being 0.79), and a 0.81 proportion of good contigs. On average, 95% of sample reads were successfully aligned to the transcriptome.

3.3.6 RNA-Sequencing

Total RNA was sequenced using the LifeTech Ion Proton platform (2013 samples, single-end reads, average length of 100 nt) or the Illumina NextSeq 500 (2015 samples, 100 nt paired-end reads)., and then we aligned reads to the transcriptome using the Salmon aligner (Patro et al. 2015). Since we are not particularly interested in differential splicing, we then collapsed the read counts per transcript down to the gene level using the Bioconductor tximport package, version 1.0.3 (Soneson et al. 2016). We collapsed the transcripts to genes in two steps. We first summed counts from all the Trinity transcripts that were assigned to the same Trinity gene, and then selected those genes with a mean log counts/million greater than zero, attempting to remove those genes that represent either transcripts that are expressed in only a small subset of samples, or transcripts that are unlikely to be accurate. We then used the National Center for Biotechnology Information (NCBI) *Basic Local Alignment Search Tool* (blastn, version 2.4.0+) to align a representative transcript from each gene to the non-redundant (nr) database (downloaded 2016/07/08). We aligned to nr rather than using blastx against the SwissProt/UniProt database because the latter returned very few hits. We accepted any blast alignment with an E-value $< 10^{-10}$, and then used any duplicated blast hits to further collapse transcript counts to a set of unique genes (e.g. if two different Trinity transcripts aligned to the same gene according to blast, we combined read counts for those transcripts). This resulted in counts for a total of 19.3k genes, 85% of which were annotated with a GI number. Since there is very little genomic information available for Steller sea lions (or pinnipeds for that matter), we annotated sequences using the lowest E-value (probability of random alignment) regardless of species. A high proportion (40%) of our genes were annotated with the predicted Pacific walrus (*Odobenus rosmarus divergens*), and other closely related species with genomic information available were represented (9% to giant Panda (*Ailuropoda melanoleuca*), 7% to polar bear (*Ursus maritimus*), and 5% to Weddell seal (*Leptonychotes weddellii*)).

3.3.7 Comparison of gene expression

To compare counts per gene (gene expression) we utilized the Bioconductor limma package (version 3.28.21) and the *voom* function which generates precision weights for each normalized count data (Smyth 2005, Law et al. 2014). We filtered out all genes that had a consistently low expression level, because these genes tend to be highly variable, having a high noise to signal ratio. After filtering, there were 19,300 genes remaining.

We then fit an analysis of variance (ANOVA) model that includes a nuisance variable that captures the batch (2013 *vs* 2015) differences, and then made comparisons between the high and low [THg] pups. We found that after conducting a principal component analysis of these data that there were significant differences between male and female pups (Figure 3.3). Since there are known sex based differences in gene expression (*i.e.* Harley et al. 2016) we decided to remove the female pups in order to help increase power to detect differences associated with different [THg] in blood. We selected genes based on an unadjusted p-value < 0.05 and a 25% difference in expression, using the limma treat function (McCarthy and Smyth 2009), which incorporates the fold change criterion as part of the significance testing. Enrichment tests were performed using the Ingenuity Pathway Analysis (IPA, Qiagen) for canonical toxicological pathways as well as biological processes. This assessment uses a Fisher Exact Test which calculates the ratio of genes associated with a particular pathway to the total number of genes in the subset. Higher ratios are less likely to occur under the null distribution, thus they are inferred to be due to perturbations in the underlying pathway due to Hg exposure.

3.3.8 Metagenomic microbial mining

Following our analysis of comparative gene expression, we analyzed our data using ContextMap following the protocol stated in Bonfert et al. (2013). One use for ContextMap is to mine RNA-Seq data sets for evidence of microbial contamination in host biological samples (Bonfert et al. 2013). Briefly, sequenced reads were aligned in a competitive manner to the host transcriptome as well as to various microbial genomes, selecting the best alignment for each read. The basic idea underlying this analysis is

that any reads that align with higher fidelity to a microbial genome than the host genome gives some evidence that the sample was contaminated by that microbe, where the contamination could represent an infection of the animal, or simply contamination during sample procurement or processing.

Although an unbiased survey of pathogens in wildlife species might ultimately be developed, at the moment technical and bioinformatics limitations prevent such microbial mining from being truly unbiased. Firstly, there is incomplete genomic information available for many potential bacterial and viral pathogens that Steller sea lions could be exposed to, and without the aid of targeted PCR to amplify genes of interest the RNA-Seq mining technique is not guaranteed to pick out gene sequences that might be used to identify or distinguish pathogens. Secondly, this technique is not suited to identify novel pathogens (*i.e.* those without genomes publicly available) due to the nature of the mapping algorithms.

Considering the above and exploring proof of principle, we instead focused on potential pathogens that a) had full genomes available on NCBI, and b) have been known to be present in Steller sea lions or closely related pinnipeds. We analyzed our sequences for 7 potential pathogens including *Mycoplasma haemolamae* (Volokhov et al. 2011), *Coxiella burnetti* (Minor et al. 2013), *Streptococcus bovis* and *Streptococcus phocae* (Lee et al. 2015), *Brucella pinnipedialis* (Burek et al. 2005), Phocine distemper virus (Zarnke et al. 2006, Goldstein et al. 2009), and Influenza A (Danner et al. 1998, Nielsen et al. 2001).

3.4 Results

3.4.1 RNA quality and QA/QC

RIN values generated from the Bioanalyzer 2100 were generally > 8 (mean 9.2 ± 0.6 standard deviation), indicating RNA-quality is sufficient for next-generation sequencing experiments (Table 3.1). One sample had a RIN value of 7.10, indicating some degradation; therefore, this sample was not included in the final analysis. Samples extracted in 2013 had a higher mean RIN than samples extracted in 2015 (9.6 versus 8.7, $p<0.01$ using student's t-test). There is evidence that degradation of RNA can

influence comparisons of gene expression (*i.e.* Opitz et al. 2010), however these findings were related to more highly degraded RNA (RIN 5-8).

3.4.2 Total mercury concentration and body condition

Mean [THg] are presented in wet weight (ww) for each treatment group for Agattu Island in Table 3.2. Mean [THg] in whole blood did not vary significantly between the 2013 and 2015 for Agattu Island (Tukey HSD, $p > 0.05$). The body condition index estimates for animals from Agattu Island did not vary significantly between the two years (Tukey HSD, $p=0.33$), although animals from Ulak Island had higher CI than grouped animals from Agattu Island ($p=0.03$). Body condition indexes ranged from 45 to 64 (geometric mean=52, s.e.=0.84), which is comparable to values found in Rea (1995). No individuals were determined to be statistical outliers based on one-tailed Dixon's Q-test. After robust linear regression to examine the relationship between [THg] and CI, we found that the 95% confidence interval for the slope bounded 0, thus we do not have evidence to suggest that [THg] varied with the body condition for these animals.

3.4.3 Comparison of gene expression

After removing female pups and filtering out genes with little or no expression, we had no evidence that any genes were differentially expressed after accounting for multiple comparisons. Recognizing that this study is most likely under-powered due to various constraints, we relaxed our selection criteria to an unadjusted $p < 0.05$, and a 25% change in expression, after which 104 genes were identified as differentially expressed. We attempted to identify pathways that are affected by Hg exposure using the Core Analysis feature of the Ingenuity Pathway Analysis software, but this analysis did not reveal any significant enrichment of specific pathways or biological processes. Unfortunately, many of the 104 genes were not recognized by IPA, and only about half of the genes belonged to any ontological pathway. Interestingly, 90 (87%) of the differentially expressed genes were downregulated with respect to the high [THg] group (Figure 3.4). The distribution of all genes shows roughly equal proportions of up-

and down-regulation between the two groups (48% and 52%), but a $\chi 2$ test for proportions indicates that down-regulated genes are significantly over-represented in our set of differentially expressed genes ($p < 0.01$).

3.4.4 ContextMap microbial mining

For most samples there was little or no evidence for microbial contamination. A few had reads that were aligned to microbial genomes, but these reads were either rare (~100 out of approximately 100 million) or short reads (<20nt). One sample however had several thousand reads that aligned to the 23s rRNA region of Candidatus *Mycoplasma haemolamea* (Guimaraes et al. 2012).

3.5 Discussion

3.5.1 Application of emerging tools to Alaska wildlife population management

The application of rapidly developing modern genomics tools to study remote wildlife populations is a challenging prospect, although given the large strides that "–omics" technologies have made in the past decade it would be imprudent not to adapt and/or apply these tools for use with marine mammals. While other researchers have shown successful RNA-Seq analyses in the blood of captive marine mammals (*i.e.* Morey et al. 2016), we have shown here that these studies do not have to be limited to readily accessible individuals/populations. Indeed, much marine mammal work is done on long research cruises or at various polar "stations" where ability to ship samples or access to -80^{o}C freezers might not be feasible for several weeks or months. Additionally, the ability for the PaxGene™ blood RNA tubes to stabilize RNA even at room temperature make them feasible for some remote field work (*i.e.* land based sampling or dart biopsies) where researchers might not have access to a freezer for several hours. Implementation under remote live capture of SSL pups in this study has resulted in the acquisition of high quality RNA samples allowing us to do this investigation with the needed confidence in sample quality.

3.5.2 Comparisons of gene expression related to [THg] in blood

In our evaluation of differential gene expression between animals with high [THg] (above concentrations associated with clinical signs of neurotoxicity, (Clarkson and Magos 2006) in blood versus animals with low concentrations (below increased risk range, Health Canada, Figure 3.2) we did not find any significant pathways or processes that were enriched in male SSL pups which would indicate changes in gene expression due to Hg exposure. We offer several hypotheses to explain this result.

The first explanation is that, due to logistical and financial restrictions we were limited in scope and sample size to only 24 individuals. We further focused our analysis to account for two confounding variables –differences in gene expression due to sex (Figure 3.3) and differences between the two rookeries. There were large differences in gene expression between Agattu and Ulak Islands even after controlling for [THg], and since we cannot rule out the possibility that there were island differences driven by variables other than [THg]. For instance, distribution of sexes from our sampled animals was not equal between islands, so it is possible that sex based differences in gene expression drove differences between islands. It is also possible that there could be genetic differences between the two rookeries, although studies into genetic variability haven't found conclusive evidence for within-stock structure (Hoffman et al. 2006). We focused our differential expression analysis on male pups from Agattu Island where there was a large variability in [THg] between animals (13 samples). Our statistical power was thus limited, although even with this limitation we expected to uncover a few differences between these treatment groups. In conjunction with this explanation is the fact that gene expression, especially in whole blood, is rather variable both between and within (temporally) individuals (Whitney et al. 2003, Morey et al. 2016). In laboratory conditions researchers can control confounding variables such as diet, exercise, and exposure to pathogens which could potentially generate large scale changes in gene expression (Su et al. 2002, Jaenisch and Bird 2003). However, in wildlife populations there are a number of differences in diet and pathogen exposure that cannot be accounted for, and likely still others that have not yet been considered.

An alternative explanation is that, while concentrations of [THg] in the blood of SSL pups were high compared to some mammalian benchmarks, [THg] in marine mammals often positively correlates with concentrations of the well-known antioxidant selenium (Koeman et al. 1973, Dietz et al. 2000). Selenium and mercury have an interesting biochemical relationship, with selenium binding to mercury directly following demethylation (Hg-Se, tiemmanite) as well as acting as an antioxidant against damage from oxidative radicals caused by $MeHg^{+}$ intoxication (Berry and Ralston 2008, Khan and Wang 2009). McHuron et al. (2014) found mean TSe-THg molar ratios in whole blood of between 7 and 8 for harbor seals even in the highly contaminated San Francisco Bay, where mean [THg] in whole blood were significantly higher than concentrations found in the Aleutian Islands (0.35 μg/g ww for females and 0.44 μg/g ww for males). A previous study of Se-Hg molar ratios in Steller sea lion pup carcasses found that most had ratios of at least 0.9 in all tissues measured (they did not measure blood), although the sampling of stranded individuals is potentially not representative of the general population (Correa et al. 2014). Given the sequestration of mineralized Hg-Se, it is hypothesized that molar ratios of Se-Hg of at least one are necessary to support normal intracellular Se-based protein function (Khan and Wang 2009). There is some conflicting evidence regarding the protective effects of Se in marine mammals. Das et al. (2016) found no protective effects of Se in harbor seal leucocytes in molar ratios of 1:10 Hg:Se. It is possible that the antagonistic relationship of Hg and Se is dependent on the species in question as well as the chemical forms of each element that likely varies by tissue type (Khan and Wang 2009).

Interestingly, a further examination of the list of differentially expressed genes reveals several genes involved in immune response with demonstrated response to viral infection in other species including AIM2, DDX58, DDX60, IFIT1, MX2, OAS1, RSAD2, and SAMD9L. $MeHg^{+}$ is known to be an immunosuppressant, thus it is plausible that individuals with higher Hg might be more susceptible to infection than animals with low Hg. Since the majority of these genes were downregulated in the high Hg group, it is conceivable that some evidence of immunosuppression was seen. However since we did not

find evidence for statistical pathway enrichment, we interpret these results with caution and encourage further studies examining specific genes and measures of immune function.

Some marine mammals often present [THg] above adverse effects thresholds from model organisms, however few studies have provided conclusive evidence of Hg toxicity causing deleterious health effects in wild populations of marine mammals although several have noted associations between high Hg concentrations and various adverse outcomes (Dietz et al. 2013). For instance, associations have been found between high [THg] in tissues and higher likelihood of bacterial infection or parasite prevalence (Siebert et al. 1999), potential association with liver lesions in polar bears, or subclinical signs such as decreased levels of N-methyl-D-aspartate (NMDA) receptors in the cerebellums of polar bears. While it is difficult to link heavy metal concentrations to adverse effects, it is likely that one of the earliest signs of a biological response to high concentrations of heavy metals would be changes in gene expression. The fact that we did not see changes in gene expression associated with different concentrations of circulating THg perhaps suggests that chemical interactions (*i.e.* Se-Hg interactions) obviate biological responses to Hg via the mineralization of Hg-Se complexes and other processes. On the other hand, Gonzalez et al. (2005) suggested that the lack of observed changes in gene expression in brain tissue of zebrafish (*Danio rerio*) following exposure to $MeHg^+$ could be a contributing factor to the observed neurotoxicity of $MeHg^+$ in this tissue. In other words, the lack of response via initiation of transcription of defensive proteins (antioxidant response elements, metallothioneins, *etc.*) might correlate with toxicity in that tissue.

Our finding that down-regulated genes were over-represented in our set of differentially expressed genes is interesting because a number of reports have indicated changes in global DNA methylation associated with $MeHg^+$ exposure (Basu et al. 2014). DNA methylation in mammals occurs mainly at cytosine-guanine dinucleotides (CpG cites) which can affect the expression of specific genes as well as induce large scale changes in global expression (Basu et al. 2014). Inverse associations between global methylation and environmentally relevant Hg concentrations have been found in male polar bears

(Pilsner et al. 2010), *in vitro* rat neural stem cells (Bose et al. 2012), and the American alligator (*Alligator mississippiensis* (Nilsen et al. 2016), though others have found no association (*i.e.* chicken (*Gallus gallus*) in Basu et al. 2013). However Head (2014) suggested that DNA methylation and resulting changes in gene expression might be highly variable and species-specific, and instances of hypermethylation of certain loci following exposure to different forms of Hg have been described (Onishchenko et al. 2008, Hanna et al. 2012). This might explain why we found suppressed transcription of nearly all of our differentially expressed genes in animals with high [THg], although this will need to be confirmed with an analysis of global DNA methylation of SSL peripheral blood leukocytes in relation to THg concentrations.

3.5.3 Microbial mining of RNA-Seq data

The concept of mining RNA-Seq data from a host for microbial RNA is relatively nascent, however it could prove an important tool in the diagnosis or identification of pathogenic bacteria and viruses. In their development of the ContextMap alignment algorithm, Bonfert et al. (2013) showed that their method could detect the expression of HPV-18 in a sample of HeLa cells. In this study, we attempted to detect sequences of RNA which would indicate the potential presence of pathogens that are known or suspected to exist in Steller sea lions.

We found evidence to suggest that some of the animals that we sampled had sequences representative of *Coxiella burnetti, Streptococcus bovis, Streptococcus phocae, Brucella pinnipedialis,* Phocine distemper virus, or Influenza A. However, without traditional gold-standard analyses such as antibody titers or traditional PCR we cannot confirm that these individuals were afflicted by these pathogens. In one sample we had a significant number of reads align to Candidatus *Mycoplasma haemolamae*, most of the reads were aligned to the 23s rRNA portion of the genome. The presence of a hemotrophic *Mycoplasma* previously described in alpacas (*Vicunga pacos*) seems unlikely – however recently a novel hemotrophic Mycoplasma was described in California sea lions which was most closely related to Candidatus Mycoplasma haemolamae (Volokhov et al. 2011), although the sea lion species had

only 92% sequence similarity to the camelid strain. When we used blast on the sequences that had aligned to the 23s region of Candidatus M. haemolamae we found that they aligned to the *Candidatus Mycoplasma haemominutum 'Birmingham 1'* noncontiguous finished genome (89% homology; (Zhang et al. 2000).

The 23s sequences of rRNA belonging to *Mycoplasma* species may represent environmental contamination, rather than infection or exposure. Indeed, as the smallest reproducing organism some *Mycoplasma* species are annoying laboratory contaminants. However, the alignment to hemotrophic (obligate red blood cell parasite) *Mycoplasma* species suggests that environmental contaminations would be unlikely from skin, *etc.* (*i.e.* blood samples were drawn via venipuncture). Hemoplasmic *Mycoplasmas*, such as Candidatus M. hemolamae and Candidatus M. haemozalophi, are not found in environmental samples (contamination from the environment, *i.e.* soil, water, skin) due to their dependence on a host cell for survival and reproduction (Messick 2004). Although it is interesting to consider the physiological and population level impacts of detecting a hemotrophic *Mycoplasma* in the blood of Steller sea lions, without confirmation of PCR or RT-PCR we simply suggest that RNA-Seq can be a useful tool in disease surveillance in wildlife species.

The discovery of 23s regions aligning closely to *Mycoplasma* spp. is interesting from population management as well as human health perspectives. Marine mammal mortality events have been associated with *Mycoplasma* spp. (Kirchhoff et al. 1989, Ruhnke and Madoff 1992) and infection from various *Mycoplasma* are associated with respiratory diseases including pneumonia. Additionally some *Mycoplasma* spp. are zoonotic and generally thought to cause "seal finger" disease in humans, which can be common in people exposed directly to pinnipeds (Waltzek et al. 2012). In Alaska, Steller sea lions are hunted as part of a subsistence lifestyle, thus the determination of zoonotics in Alaska pinnipeds is critical from a human health standpoint.

These preliminary results from applying emerging NGS tools and pipelines to a threatened and difficult to access wildlife population suggests that— despite the difficulty of remote field work and the lack of comprehensive genomic information—NGS tools have a place in the advancement of wildlife health assessments. The ability to examine changes in gene expression in relation to environmental and morphological variables, examine evidence for pathogens, and contribute to the building of a molecular profile for an endangered species promotes RNA-Seq and other NGS tools to the frontlines of marine mammal research. Although this is a preliminary *de novo* transcriptome, we feel that with further refinement and annotation having a transcriptome for the Steller sea lion will be a useful tool in further assessments of health status and population monitoring.

3.6 Acknowledgements

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3.7 Figures

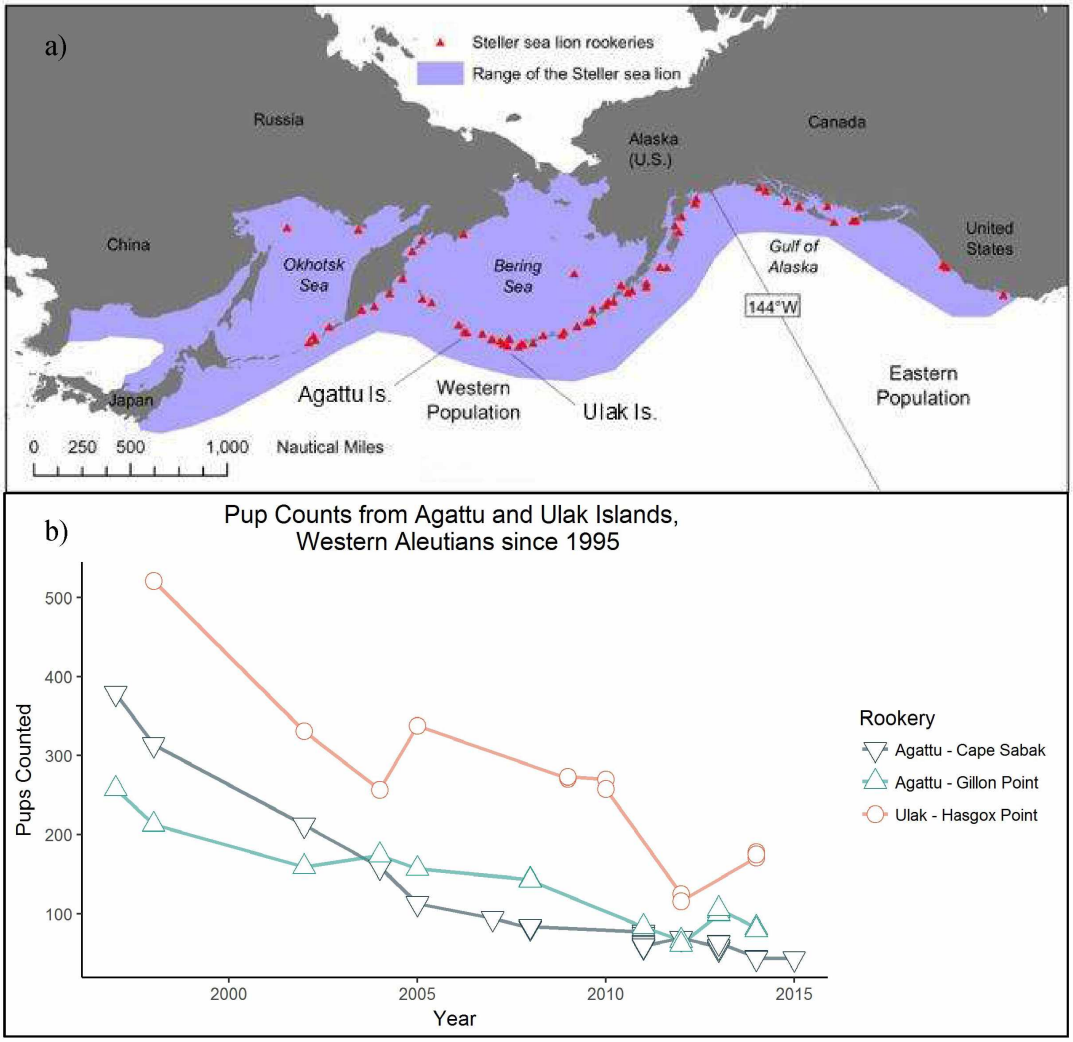

Figure 3.1 – (a) The range of Steller sea lions. The boundary between the EDPS and WDPS is shown at 144°W. The WDPS was listed as "Endangered" while the EDPS was "Threatened" in 1997, although the EDPS was delisted in 2013 following significant recovery of the population there. The WDPS is still listed as "Endangered". Agattu and Ulak Islands are highlighted in the Aleutian Island archipelago. Figure is adapted from Alaska Fisheries Science Center (www.afsc.noaa.gov). (b) Pup counts from Agattu Island (2 beaches) and Ulak Island since 1995. Data are from Fritz et al. (2015).

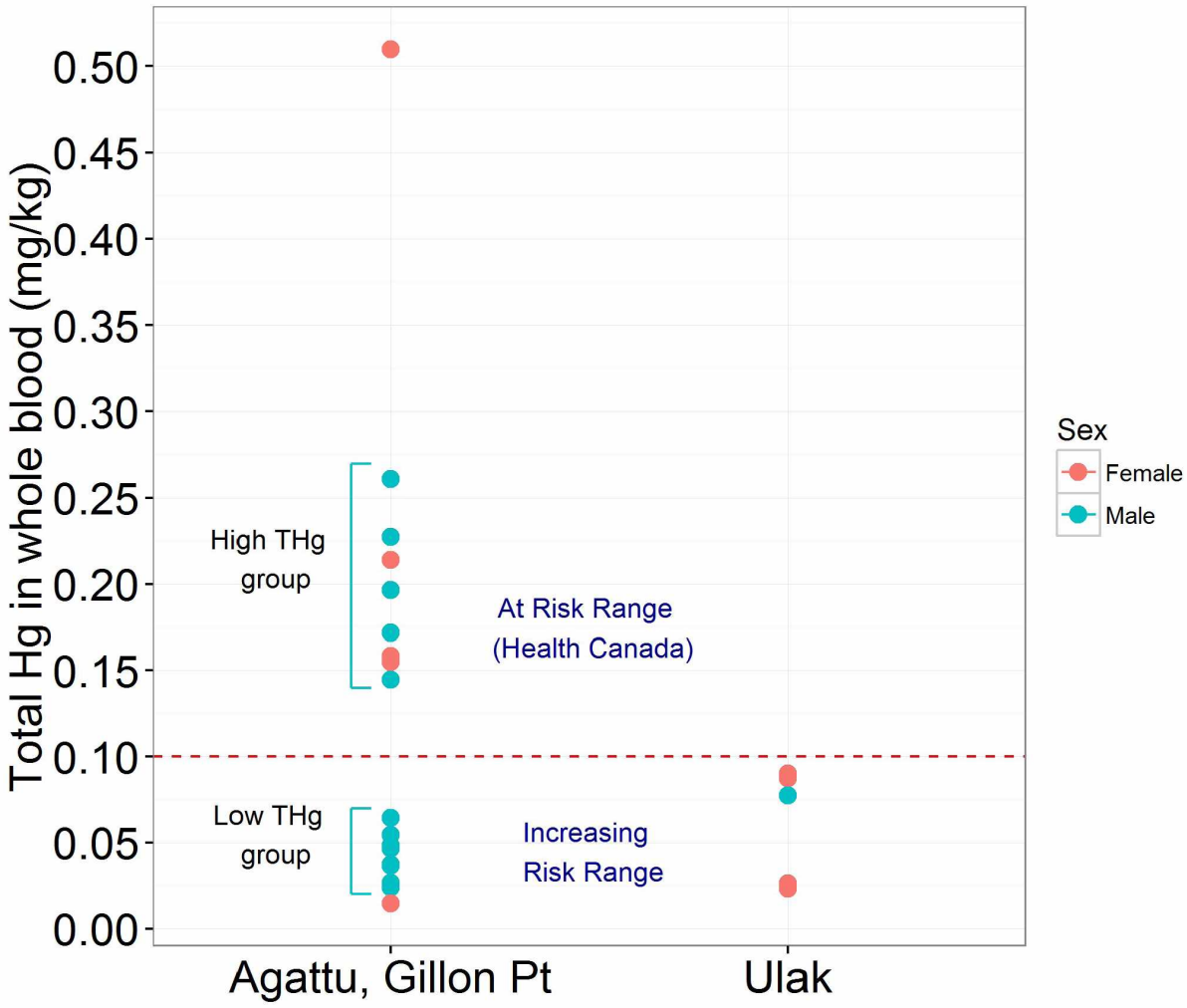

Figure 3.2 – Total mercury concentration ([THg]) in whole blood of Steller sea lion pups from Agattu and Ulak Islands included in our assessment of differential gene expression. Females were excluded from the comparative gene expression analysis in order to limit confounding sex linked genes, and since only one male was selected from Ulak Island we decided to drop this individual from the final analysis. Animals with [THg] below 0.10 µg/g ww were grouped in the low [THg] group, while animals with concentrations above 0.10 µg/g ww were grouped in the high [THg] group.

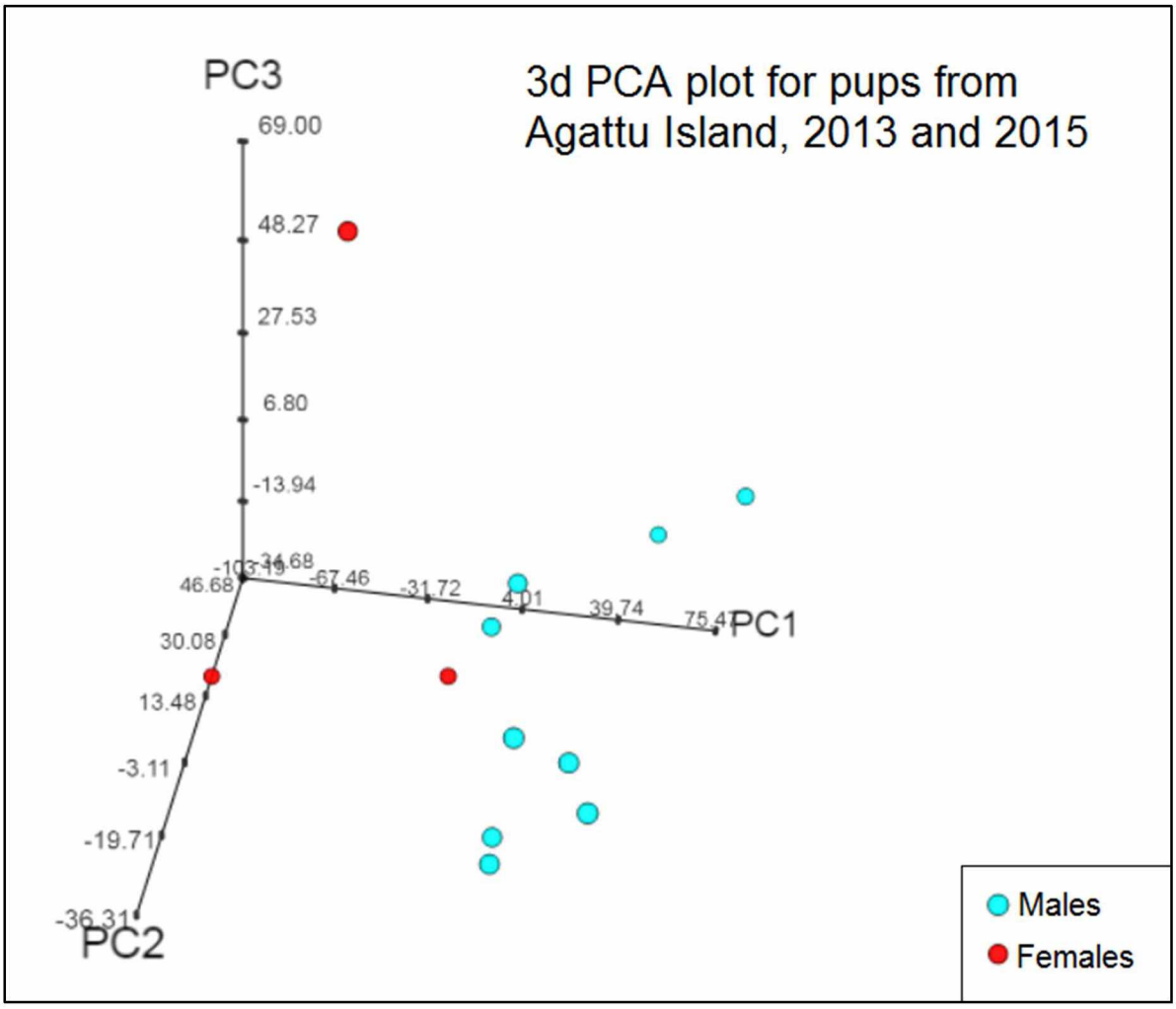

Figure 3.3 – Three-dimensional PCA plot showing differences between male and female pups.

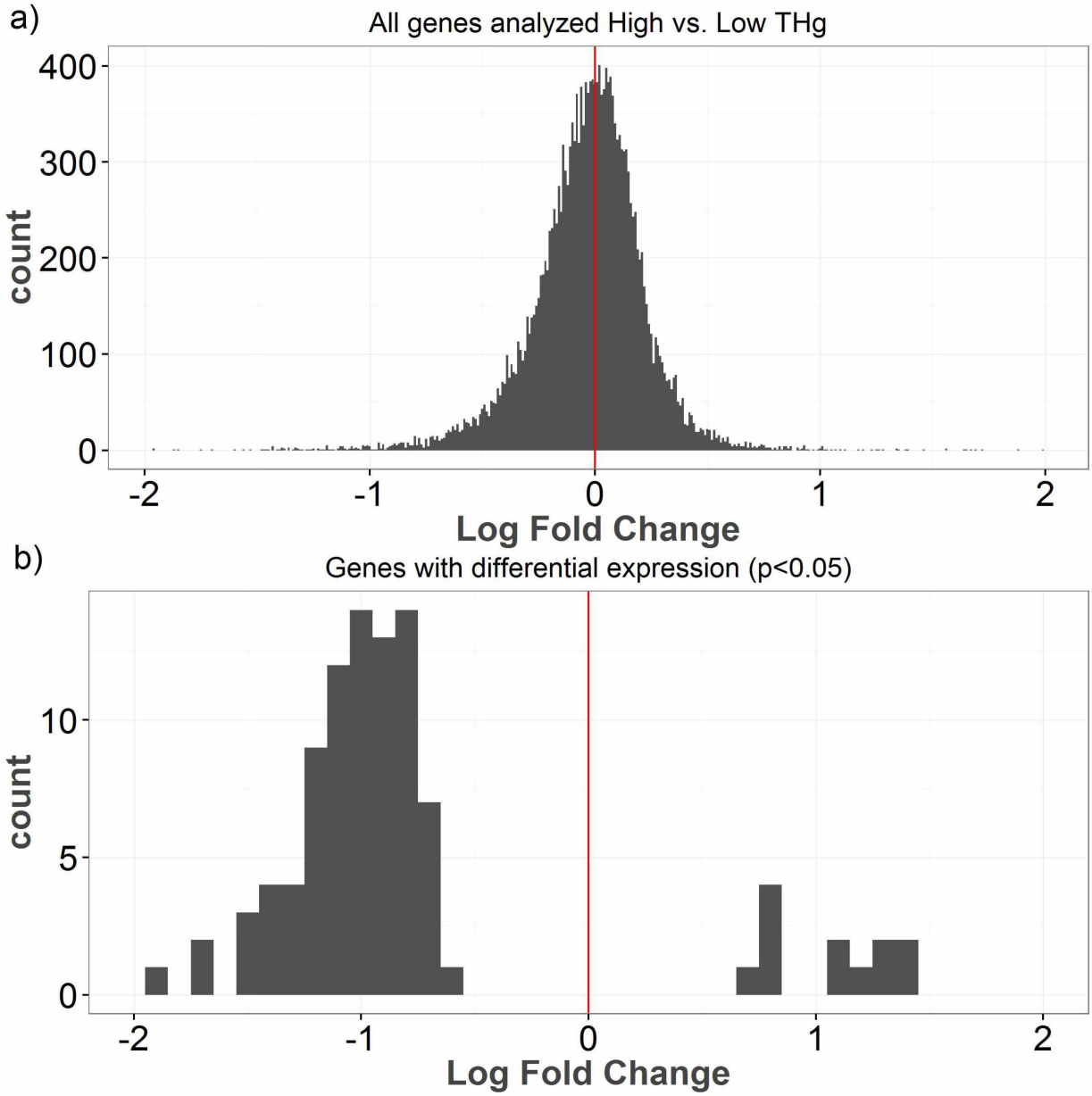

Figure 3.4 - A comparison of genes between animals with high [THg] and animals with low [THg]. (a) A histogram of all genes is plotted here according to the log fold change (LFC) difference between the two groups. (b) Of the 104 genes that were identified by the non-conservative model, 90 were downregulated with respect to the high [THg] group.

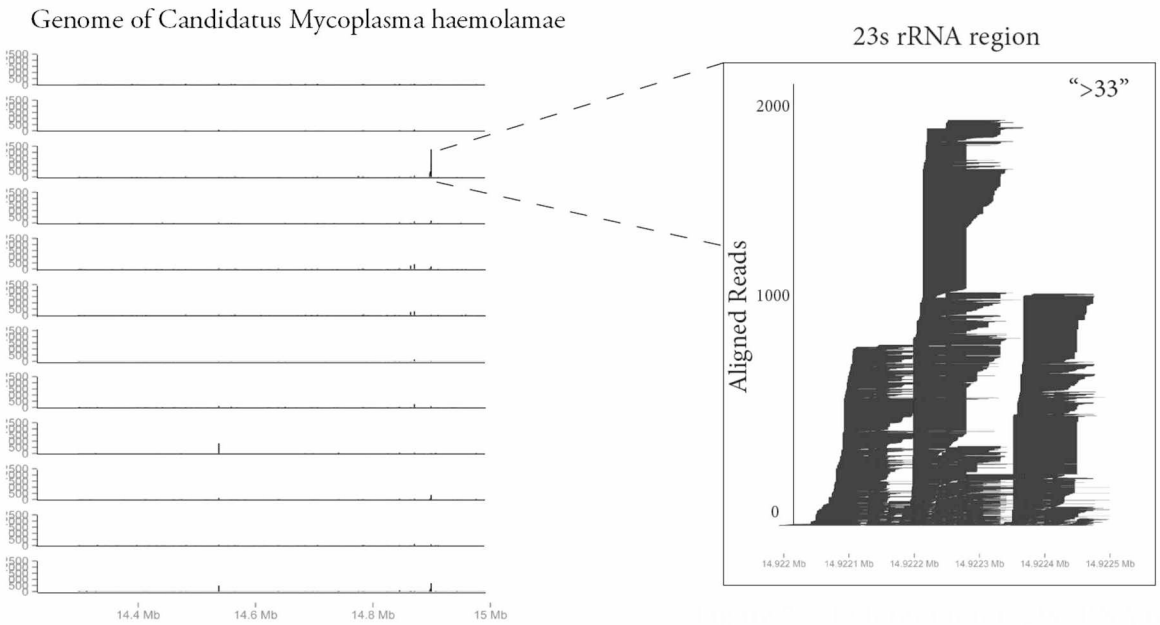

Figure 3.5 – Pileup plots of sequences aligned by ContextMap to the genome of Canidatus Mycoplasma haemolamae. One sea lion pup, ">33" showed a large number of sequences aligning to the 23s rRNA region of Candidatus M. haemolamae.

3.8 Tables

Table 3.1 – Sample description, blood [THg] values, study group information, and body condition scores for the animals used in this study. CI index is an estimate of body condition based on a ratio of girth to length as utilized by Rea et al. (2016).

Brand ID	Rookery/ Island	Year	Sex	[THg] WB (ppm, ww)	CI Index (body condition)	RIN
~61	Agattu	2013	Male	0.261	61	9.5
~60	Agattu	2013	Male	0.172	66	9.4
~69	Agattu	2013	Female	0.155	66	9.4
~80	Agattu	2013	Male	0.037	72	9.5
~72	Agattu	2013	Male	0.024	69	9.4
~85	Agattu	2013	Female	0.015	75	9.7
>34	Ulak	2013	Female	0.090	67	9.4
>30	Ulak	2013	Female	0.088	77	9.8
>351	Ulak	2013	Female	0.077	70	9.6
>42	Ulak	2013	Male	0.077	68	9.9
>31	Ulak	2013	Female	0.026	75	9.9
>33	Ulak	2013	Female	0.023	74	9.5
~126	Agattu	2015	Female	0.510	66	9.2
~121	Agattu	2015	Male	0.227	74	9.1
~124	Agattu	2015	Female	0.214	70	8.3
~113	Agattu	2015	Male	0.197	76	8.5
~128	Agattu	2015	Female	0.159	80	7.1
~139	Agattu	2015	Male	0.145	73	8.6
~122	Agattu	2015	Male	0.064	65	8.6
~115	Agattu	2015	Male	0.054	70	9.7
~141	Agattu	2015	Male	0.048	69	8.6
~135	Agattu	2015	Male	0.047	70	8.8
~154	Agattu	2015	Male	0.037	70	8.7
~145	Agattu	2015	Male	0.027	75	9.7

WB=whole blood
ww=wet weight
RIN=RNA Integrity Number

Table 3.2 - Mean THg concentrations and standard error for each treatment group.

Island	Year	Group	[THg] in WB (ppm)	SE	N
Agattu	2013	High Hg	0.22	0.05	2
Agattu	2013	Low Hg	0.03	0.01	2
Agattu	2015	High Hg	0.19	0.02	3
Agattu	2015	Low Hg	0.05	0.01	6

WB=whole blood
SE =standard error
N=number of samples

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3.10 Appendix B

Table 3.B-1 – The list of Trinity genes that were identified as significantly differentially expressed between male pups with high [THg] and male pups with low [THg] in whole blood.

Table 3.B-1 (cont.) GI=GenInfo Identifier, FC=fold change

GI	Title	Symbol	logFC	t	p-value
585194816	PREDICTED: Leptonychotes weddellii absent in melanoma 2 (AIM2), mRNA	AIM2	-1.33	-3.32	0.00
585202536	PREDICTED: Leptonychotes weddellii ataxin 3 (ATXN3), transcript variant X1, mRNA	ATXN3	-0.89	-1.92	0.04
472370154	PREDICTED: Odobenus rosmarus divergens coiled-coil domain containing 40 (CCDC40), mRNA	CCDC40	-1.39	-2.35	0.02
299116136	Homo sapiens cut like homeobox 2 (CUX2), RefSeqGene on chromosome 12	CUX2	-0.80	-1.86	0.04
472344975	PREDICTED: Odobenus rosmarus divergens DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 (DDX58), mRNA	DDX58	-0.88	-2.34	0.02
823392067	PREDICTED: Odobenus rosmarus divergens DEAD (Asp-Glu-Ala-Asp) box polypeptide 60 (DDX60), mRNA	DDX60	-1.30	-1.97	0.04
1003108906	Neophocaena asiaeorientalis asiaeorientalis MHC class II antigen (DRA), MHC class II antigen (DRB), MHC class II antigen (DQA), and MHC class II antigen (DQB) genes, complete cds	DQB	1.34	3.01	0.00
958796890	PREDICTED: Equus asinus hyaluronan synthase 3 (HAS3), transcript variant X1, mRNA	HAS3	-1.16	-2.55	0.01
823430685	PREDICTED: Odobenus rosmarus divergens interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), mRNA	IFIT1	-1.43	-2.00	0.04
859957743	PREDICTED: Mustela putorius furo interferon-induced protein with tetratricopeptide repeats 1B (IFIT1B), RNA	IFIT1B	-1.19	-2.03	0.03
752442036	PREDICTED: Ailuropoda melanoleuca immunity-related GTPase family M protein 1-like (LOC100464289), transcript variant X2, mRNA	LOC100464289	-1.08	-1.97	0.04

Table 3.B-1 (cont.) GI=GenInfo Identifier, FC=fold change

GI	Title	Symbol	logFC	t	p-value
472376407	PREDICTED: Odobenus rosmarus divergens tripartite motif-containing protein 34 (LOC101363249), mRNA	LOC101363249	-0.96	-2.31	0.02
859946248	PREDICTED: Mustela putorius furo tripartite motif-containing protein 5-like (LOC101675922), transcript variant X1, mRNA	LOC101675922	-1.53	-2.47	0.01
859957748	PREDICTED: Mustela putorius furo interferon-induced protein with tetratricopeptide repeats 2 (LOC101676809), mRNA	LOC101676809	-0.63	-1.94	0.04
585188505	PREDICTED: Leptonychotes weddellii interferon-induced GTP-binding protein Mx2-like (LOC102729981), mRNA	LOC102729981	-1.75	-2.17	0.03
585196675	PREDICTED: Leptonychotes weddellii E3 ubiquitin-protein ligase RNF213-like (LOC102737349), mRNA	LOC102737349	-1.20	-2.23	0.02
664745254	PREDICTED: Equus przewalskii uncharacterized LOC103558636 (LOC103558636), ncRNA	LOC103558636	-0.99	-1.96	0.04
671029102	PREDICTED: Ursus maritimus uncharacterized LOC103677868 (LOC103677868), transcript variant X4, ncRNA	LOC103677868	-1.23	-2.88	0.01
752440221	PREDICTED: Ailuropoda melanoleuca uncharacterized LOC105234694 (LOC105234694), ncRNA	LOC105234694	-0.73	-1.79	0.05
752431497	PREDICTED: Ailuropoda melanoleuca uncharacterized LOC105241362 (LOC105241362), ncRNA	LOC105241362	0.74	2.42	0.01
859771327	PREDICTED: Mustela putorius furo uncharacterized LOC106005160 (LOC106005160), transcript variant X4, misc_RNA	LOC106005160	-0.78	-2.11	0.03
181338224	Homo sapiens SOS Ras/Rac guanine nucleotide exchange factor 1 (SOS1), RefSeqGene (LRG_754) on chromosome 2	LRG_754	-3.79	-2.88	0.01
823412314	PREDICTED: Odobenus rosmarus divergens methionine sulfoxide reductase B1 (MSRB1), transcript variant X2, mRNA	MSRB1	-3.39	-2.42	0.02

Table 3.B-1 (cont.) GI=GenInfo Identifier, FC=fold change

GI	Title	Symbol	logFC	t	p-value
472374366	PREDICTED: Odobenus rosmarus divergens MX dynamin-like GTPase 2 (MX2), mRNA	MX2	-1.03	-1.85	0.04
545542529	PREDICTED: Canis lupus familiaris nudix (nucleoside diphosphate linked moiety X)-type motif 18 (NUDT18), transcript variant X2, mRNA	NUDT18	1.38	4.58	0.00
145699428	Equus caballus 2'-5' oligoadenylate synthetase 1 (OAS1) gene, complete cds	OAS1	-0.80	-1.80	0.05
671004539	PREDICTED: Ursus maritimus peptidyl arginine deiminase, type II (PADI2), partial mRNA	PADI2	-1.08	-2.44	0.01
472344504	PREDICTED: Odobenus rosmarus divergens poly (ADP-ribose) polymerase family, member 14 (PARP14), mRNA	PARP14	-0.78	-1.91	0.04
1042779830	Homo sapiens poly(ADP-ribose) polymerase family member 14 (PARP14), RefSeqGene on chromosome 3	PARP14	-0.85	-1.96	0.04
671031169	PREDICTED: Ursus maritimus poly (ADP-ribose) polymerase family, member 9 (PARP9), transcript variant X4, mRNA	PARP9	-0.80	-1.98	0.03
823422885	PREDICTED: Odobenus rosmarus divergens phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma) (PLA2G7), mRNA	PLA2G7	-2.04	-2.32	0.02
671031828	PREDICTED: Ursus maritimus promyelocytic leukemia (PML), transcript variant X11, mRNA	PML	-0.65	-1.90	0.04
755727732	PREDICTED: Felis catus RIO kinase 1 (RIOK1), transcript variant X1, mRNA	RIOK1	-0.78	-1.85	0.04
752410091	PREDICTED: Ailuropoda melanoleuca radical S-adenosyl methionine domain containing 2 (RSAD2), mRNA	RSAD2	-1.43	-1.90	0.04
823394965	PREDICTED: Odobenus rosmarus divergens sterile alpha motif domain containing 9-like (SAMD9L), mRNA	SAMD9L	-1.12	-2.32	0.02

Table 3.B-1 (cont.) GI=GenInfo Identifier, FC=fold change

GI	Title	Symbol	logFC	t	p-value
585196947	PREDICTED: Leptonychotes weddellii short coiled-coil protein (SCOC), mRNA	SCOC	-1.70	-2.58	0.01
859936674	PREDICTED: Mustela putorius furo smoothelin-like 1 (SMTNL1), mRNA	SMTNL1	-1.08	-2.55	0.01
859781015	PREDICTED: Mustela putorius furo TBC1 domain family, member 16 (TBC1D16), transcript variant X4, mRNA	TBC1D16	-0.96	-1.89	0.04
585174378	PREDICTED: Leptonychotes weddellii tripartite motif containing 17 (TRIM17), mRNA	TRIM17	1.07	1.85	0.04
585179340	PREDICTED: Leptonychotes weddellii tryptophanyl-tRNA synthetase (WARS), mRNA	WARS	-0.88	-2.20	0.02
826310099	PREDICTED: Propithecus coquereli tryptophanyl-tRNA synthetase (WARS), transcript variant X2, mRNA	WARS	-1.18	-1.87	0.04
22023820	Zalophus californianus MHC class II antigen (Zaca-DQB) mRNA, Zaca-DQB*10 allele, complete cds	Zaca-DQB	4.67	2.65	0.01
6850244	Homo sapiens 12q24.1-118.9-120.5 BAC RP11-256L11 (Roswell Park Cancer Institute Human BAC Library) complete sequence		1.09	2.43	0.01
9187146	Human DNA sequence from clone GS1-174L6 on chromosome 1, complete sequence		-1.47	-2.07	0.03
13234854	Human DNA sequence from clone RP11-168P13 on chromosome 13, complete sequence		-1.85	-2.80	0.01
14578062	Homo sapiens 4 BAC RP11-662D13 (Roswell Park Cancer Institute Human BAC Library) complete sequence		-1.06	-1.85	0.04
18958737	Homo sapiens chromosome 3 clone RP11-344C13, complete sequence		-0.96	-1.89	0.04
20338803	Human DNA sequence from clone RP4-740C4 on chromosome 1, complete sequence		-0.75	-2.11	0.03
26291631	Homo sapiens chromosome 11, clone CTD-2609K8, complete sequence		-0.96	-2.11	0.03
29568021	Homo sapiens chromosome 19 clone CTD-2562J15, complete sequence		-1.09	-2.35	0.02
115271022	Canis Familiaris chromosome 16, clone XX-49O13, complete sequence		0.83	1.82	0.05
148923012	Canis familiaris chromosome 21, clone XX-313P24, complete sequence		-1.34	-2.90	0.01
190341157	Canis familiaris chromosome 1, clone XX-309C2, complete sequence		-1.09	-1.94	0.04

Table 3.B-1 (cont.) GI=GenInfo Identifier, FC=fold change

GI	Title	Symbol	logFC	t	p-value
242101449	Equus caballus microsatellite DNA, locus ABGe17652		-0.90	-1.79	0.05
425906353	Elephas maximus borneensis microsatellite Em_00364 sequence		1.34	2.13	0.03

In addition to the genes presented here there were 49 genes which Trinity found to be differentially expressed ($p<0.05$) but did not align to known sequences in the NCBI database. These sequences were not used in further pathway analysis.

Chapter 4 - **Using carbon and nitrogen stable isotope modeling to assess mercury exposure for pregnant women in Baja California Sur, Mexico**[5]

[5]Harley, J.R., R. Gaxiola-Robles, T. Zenteno-Savín, A. Thiede, T.M. O'Hara. In preparation. Using carbon and nitrogen stable isotope modeling to assess mercury exposure for pregnant women in Baja California Sur, Mexico. EcoHealth.

4.1 Abstract

Previous studies of mercury in pregnant women in La Paz, Baja California Sur (BCS), Mexico found some individuals had concentrations of total mercury ([THg]) above some thresholds of concern set by health agencies. Concentrations of [THg] were associated with fish and seafood consumption and other factors, although it was unclear which marine diet items could potentially be contributing to the concentrations observed. We examined [THg] and monomethylmercury ([$MeHg^+$]) in the hair of 70 pregnant women from BCS as well as in diet items including fish, shellfish, and staple items (rice, beans, corn, and flour). Concentrations of Hg species were low in staple foods and ranged from below detection limit to 5.71parts per billion (ppb) wet weight. Geometric mean in hair [THg] was 658 ppb and [$MeHg^+$] was ppb, which were lower than previous reports. In order to examine potential contribution of Hg species to the diet of study participants, we also measured stable isotopes of carbon and nitrogen. We employed a Bayesian stable isotope mixing model to investigate the proportion of fish and seafood in the isotopic profiles of archived hair samples of these pregnant women. The largest contributors to the diet of the study participants were corn and rice, and our analysis of fish contribution to diet varyingly agreed with the self-reported fish consumption. Overall, this technique has the ability to discriminate potential sources of Hg from a diverse diet.

4.2 Introduction

Mercury (Hg) is a global pollutant. Human exposure to monomethylmercury ($MeHg^{+}$) is generally through the consumption of freshwater and marine fish (vertebrates) and seafood[6] (invertebrates) (Clarkson and Magos 2006). Inorganic mercury (Hg^{2+}) is poorly absorbed by mammals while $MeHg^{+}$ tends to bioaccumulates and biomagnify through trophic levels (Clarkson and Magos 2006, Eagles-Smith et al. 2016). Aquatic environments typically have higher concentrations of $MeHg^{+}$ due to the methylation of inorganic Hg species which occurs via sulfate and iron-reducing bacteria in hypoxic or anaerobic environments such as those found in marine or freshwater sediment (Hu et al. 2013).

Methylmercury is especially neurotoxic to the developing fetus and neonate as it crosses the placental barrier and can trigger apoptotic signaling and loss of neurons through a variety of pathways (Ceccatelli et al. 2010). The apoptotic signaling pathway, at least in some neural tissues, appears to be initiated as a result of mitochondrial permeability disruption caused by an abundance of reactive oxygen species (ROS) as well as a disruption of calcium (Ca^{2+}) homeostasis; two established consequences of $MeHg^{+}$ exposure (Ceccatelli et al. 2010, Sokolowski et al. 2011). The disruption of cation homeostasis has multifaceted effects, and appears to directly influence cell cycling resulting in neuronal hypoplasia (Faustman et al. 2002). Interestingly, transport of $MeHg^{+}$ throughout the body and across the placental and blood-brain barrier appears to be mediated by innate protein transporters (Kerper et al. 1992, Simmons-Willis et al. 2002). Since $MeHg^{+}$ has a demonstrated affinity for sulfhydryl groups (R-SH), it readily binds to cysteine (Cys) residues. This $MeHg^{+}$-cysteine complex mimics the molecular structure of methionine (Met), which is transported via neutral amino acid carriers such as L-type large neutral amino acid transports (LAT, Kerper et al. 1992; Simmons-Willis et al. 2002).

Although the neurotoxicity of $MeHg^{+}$ in animal models and humans is well documented (Clarkson and Magos 2006, Bernhoft 2012) there is considerable disagreement and conflicting data

[6] In Baja California the term *marisco*, which translates to seafood, describes various invertebrate species such as shrimp, crabs, clams, and scallops.

regarding long term effects of fish consumption during pregnancy (Sheehan et al. 2014, Myers et al. 2015). Of particular interest are longitudinal studies that attempt to correlate estimated *in utero* mercury exposure to developmental milestones and cognitive abilities, such as the Seychelles Child Development Study, which has tracked 779 children born in 1989-1990. Residents of the Seychelles Islands consume large quantities of fish with $MeHg^+$ concentrations that are comparable to commercially available fish consumed in the United States (Davidson et al. 2006), although median concentrations of maternal and child hair $MeHg^+$ were an order of magnitude higher than those of frequent fish consumers in the U.S. (Davidson et al. 1998, McDowell et al. 2004). Despite the relatively high concentration of fetal exposure, there have been only a few reports of negative associations between developmental outcomes and these $MeHg^+$ concentrations (Myers et al. 2003, van Wijngaarden et al. 2013), and the most recent examination of the original study cohort (at 22-24 years) found no convincing evidence for adverse effects of fish consumption during pregnancy (van Wijngaarden et al. 2017).

Although there is some evidence that low-dose $MeHg^+$ exposure via fish consumption is correlated with developmental deficits in children, the lack of observed toxicity in longitudinal studies (i.e. Seychelles) may be due, at least in part, to nutritional components of fish which may counteract $MeHg^+$ induced toxicity and may support proper fetal development (Egeland and Middaugh 1997, Gribble et al. 2016, Oken et al. 2016). Fish contain high concentrations of antioxidants including selenium (Se) and long chain omega-3 polyunsaturated fatty acids (PUFAs) which are essential for normal brain development and function. In an animal model, pups from pregnant mice concurrently exposed to both $MeHg^+$ and Se did not show the same developmental deficits as mice exposed solely to $MeHg^+$. Several studies have found a positive correlation between fish consumption and developmental endpoints (Sakamoto et al. 2004, Oken et al. 2008, Mahaffey et al. 2011, Strain et al. 2012), although some have noted that selecting fish with high concentrations of omega-3 PUFAs and low concentrations of $MeHg^+$ and other toxicants (i.e. organohalogens, biotoxins) would be optimal (Egeland and Middaugh 1997, Gribble et al. 2016). In humans studies have noted benefits in birth and developmental outcomes with the

consumption of fish oil supplements during pregnancy (Olsen et al. 1992, Sakamoto et al. 2004, Dunstan et al. 2008).

The two states of Baja peninsula in Mexico (Baja California, and Baja California Sur, BCS, Figure 4.1) are bordered by the Pacific Ocean and the Gulf of California (Sea of Cortez) which together constitute the majority of fisheries production for the country (Erisman et al. 2010). Fish and seafood are important dietary components for many residents of BCS, although the frequency and identity of species consumed are not well known (Gaxiola-Robles et al. 2013, 2014). Initial findings examining total mercury concentrations ([THg]) in the hair of a cohort of pregnant women found that a sizeable proportion (54 out of 75 women) had hair [THg] above the EPA advisory guideline of 1,000 parts per billion (ppb) and some (6 of 75 women) had concentrations above 5,000 ppb (Gaxiola-Robles et al. 2014). In a companion study, enrichment of nitrogen isotope ^{15}N ($\delta^{15}N$) used as an indicator of fish consumption was associated with higher [THg], although interestingly lower mean [THg] was seen in the women who reported consuming fish and shellfish most frequently (Bentzen et al. 2014). Other factors significantly associated with [THg] which could play a role were passive tobacco exposure and body mass index.

Stable isotopes of carbon ($\delta^{13}C$) and nitrogen are used to reconstruct diet through multidimensional mixing models (Moore and Semmens 2008). These models are based on the principles that lighter isotopes of C and N are more easily incorporated into biochemical processes, and thus preferentially excreted resulting in tissular enrichment of the heavier isotope. Across trophic levels this results in significant enrichment of N^{15} (usually 3-4‰, known as the trophic enrichment factor TEF) and smaller but measurable enrichment of C^{13} (1-2‰ per trophic level in most mammalian tissues) with increasing trophic levels (Kelly 2000). Food web movement of carbon stable isotopes in marine systems is somewhat complicated by the fact that different environments (i.e. benthic versus pelagic) display different $\delta^{13}C$ values in primary producers (France 1995).

In wildlife, stable isotope mixing models (SIMMs) are increasingly utilized as tools for assessing the diet and ecology of difficult to observe species and habitats (Parnell et al. 2013, Phillips et al. 2014). In human populations C and N stable isotopes have been validated for use as a assessment dietary intake of sugars (Nash et al. 2014) and marine items (O'Brien et al. 2017). Although it might seem imprudent to conduct chemical diet assessments in situations where dietary recall and communication with participants is possible, self-reported consumption and diet recalls may be of limited use and are occasionally conflicting with chemical analyses (O'Brien 2015). In addition, Hg in hair represents several weeks or months of accumulation based on the length analyzed (growth rate ~1cm/month, (LeBeau et al. 2011)), and dietary assessments aimed at assessing consumption over long time periods (i.e. food frequency questionnaires, FFQs) have multiple shortcomings and inaccuracies and their merit is highly debated (Shim et al. 2014). Thus, a more detailed chemical feeding ecology based assessment of Hg exposure in conjunction with dietary recall data is warranted (Bentzen et al. 2014).

Reports of Hg concentrations in marine fish landed and consumed from Pacific waters in Mexico have mainly been focused on top predatory fish, some of which had concentrations higher than the FDA action level of 1,000 ppb wet weight (García-Hernández et al. 2007, Ruelas-Inzunza et al. 2008, Barrera-García et al. 2012). Mexico ranks as the 6th largest producer and 6th largest importer of shark products, with a large portion of the meat destined for domestic consumption (Dent and Clarke 2015). Per capita consumption estimates of shark are relatively low compared to other categories of seafood; however $MeHg^+$ concentrations in large predatory taxonomic groups such as sharks and tunas can be several orders of magnitude higher than more commonly consumed items such as shrimp and tilapia. It is important to note that not all fish with high Hg concentrations are large predatory species ; some fish appear to store $MeHg^+$ in muscle over liver (based on concentration) and in long lived species this can lead to relatively high [$MeHg^+$] (Harley et al. 2015). At the time of writing we could find no comprehensive surveys of frequency of species consumption for fish consumers in this region, although landing and per capita

consumption data for the country are compiled by the Comisión Nacional de Acuacultura y Pesca (CONAPESCA).

Some of the most frequently consumed fish in Mexico are from aquaculture (CONAPESCA). The potential for Hg bioaccumulation in aquaculture fish is not well characterized, and appears to be extremely variable based on feed supply (Kelly et al. 2008, Bhattacharyya et al. 2010). For instance, one study found that Hg concentration in sewage fed aquaculture (a proposed method for water treatment as well as fish production, i.e. Jana, 1998) resulted in higher Hg concentrations than control sites, and sewage fed Mozambique tilapia (*Tilapia mossambicus*) had mean concentrations at or above the World Health Organization limit for consumption (500 ppb ww). However, farmed tilapia is generally found to have low concentrations of Hg (see *vignette* below).

Fish consumption is almost always considered the primary route of Hg exposure for non-occupational sources and pathways (Clarkson and Magos 2006), although some reports have indicated that other dietary sources of Hg should be considered. While fish may be commonly consumed for some individuals, it is likely that consumption of staple foods derived from corn, rice, and beans are higher in both frequency of consumption and portion size. The US Food and Drug Administration (FDA) has been conducting a Total Diet Study (TDS) since 1991 in which they have analyzed over 70 non-fish food items for [THg] including items such as milk, vegetables, chicken, and beef, although only fish-based items (e.g. fish sticks) had biologically significant concentrations (EPA, 2011). However, recent findings have indicated that at least in some regions rice may contain appreciable concentrations of $MeHg^{+}$ (Barrett 2010, Rothenberg et al. 2014). Terrestrial plants are generally unlikely to bioaccumulate $MeHg^{+}$ in non-contaminated areas since Hg found in air or soil (i.e. not in biota) is generally in an inorganic form (Hg^{2+}, $Hg1^{+}$, Hg^{0}, (St. Louis et al. 2001). However, common rice farming techniques wherein paddies are flooded with water to prevent the growth of weeds favors the formation of anaerobic sediment – which in turn supports the proliferation of sulfur and iron-reducing bacteria which methylate inorganic Hg^{2+} to $MeHg^{+}$ (Qiu et al. 2008, Zhang et al. 2010). In addition to rice, $MeHg^{+}$ has also been examined in high-

fructose corn syrup (HFCS), and although the majority of samples showed non-detectable concentrations of $MeHg^+$, the authors report that HFCS manufactured in plants using mercury grade caustic soda contained [THg] high enough to be considered in dietary assessments (65 to 570 ppb wet weight, Dufault et al. 2009).

In the present study, we assessed [THg], [$MeHg^+$], and stable isotopes of C and N in 70 pregnant women from BCS. We also measured those variables in fish and seafood purchased from BCS as well as staple foods purchased and donated from the study participants. Using our stable isotope models, we examined the relative contributions of diet items using Bayesian stable isotope mixing models. We also sought to address major sources of Hg using chemical analysis, and compare those to data from self-reported dietary surveys.

4.3 Methods

4.2.1 Sample collection

Fish and seafood samples were purchased (generally as whole fish) from fish markets in La Paz between March 2013 and May 2015. Samples of corn flour, wheat flour, and tortillas were purchased from supermarkets in La Paz. Deidentified hair samples were provided from archived samples by Dr. Gaxiola-Robles (CIBNOR) collected under the approval of the Comisión Nacional de Investigación Cientifica (permit number 2016-785-013). Occipital hair samples were obtained according to the protocol established in McDowell et al. (2004). Rice and bean samples donated by each study participant were collected to reflect the type of staple items likely to be served in their own home.

A questionnaire was provided to each participant containing questions about the frequency of consumption and portion sizes of various foods including finfish, seafood (invertebrates), pork, beef, beans, rice and other staples (a questionnaire is provided as supplemental material). The form also contained questions regarding the participant's occupation, smoking habits, dental amalgams, potential

pesticide exposure, and other questions potentially relevant to Hg exposure. Archived questionnaire data (deidentified) matched to the hair samples analyzed.

4.2.2 Sample processing

Hair samples were washed in order to remove external surface contamination using Triton X-100 detergent and Milli-Q water (Millipore, Bedford, Maryland, USA). Purchased whole fish remained frozen in a cooler until arrival at the University of Alaska Fairbanks (UAF), at which point they were placed in a freezer at -20°C until processing. Once thawed, morphometric measurements were obtained for each fish/invertebrate including length and mass. For invertebrates, total mass was recorded (including exoskeleton) and length was measured as carapace width at the widest point. Species identification was done via morphometric examination and consultation with local fish biologists. For the green crab (*Callinectes bellicosus*) samples, the frozen specimens were in poor condition upon their arrival at UAF and their claws were separated from the main body –however since muscle from both parts are consumed these were analyzed independently. Sex was not determined since most of the fish purchased from the markets were without internal organs, making visual sex identification impossible. A small portion of the cranial fillet was obtained from fish samples (1-6 grams) with the skin removed. From the chocolate clam (*Megapitaria squalida*) samples of the mantle, foot, and abductor muscle were obtained, and from the crabs muscle samples were obtained from the carapace (peropodal) and claw (cheliped). All samples were placed into Whirl-Paks (Nasco, Fort Atkinson, Wisconsin, USA) prior to analysis.

4.2.3 Freeze drying and homogenization

Samples including finfish, seafood, tortillas, and hair were freeze dried in order to facilitate Hg and stable isotope measurement. Samples in Whirl-Paks were placed into glass vacuum jars and were freeze-dried using a Labconco FreeZone 6 Liter (Labconco, Kansas City, Missouri, USA). Samples were freeze-dried for 36-78 hours depending on the water content. In the case of finfish, seafood, and tortilla samples percent moisture was calculated as the proportion of sample mass lost during freeze drying.

In order to increase reproducibility and precision all samples were homogenized using a steel-ball Cryomill (Retsch Inc, Newton, Pennsylvania, USA) for 1-2 minutes at 25 Hz. Grinding equipment was washed using Triton-X detergent, rinsed with Milli-Q water and dried thoroughly to prevent cross contamination.

4.2.4 Total mercury analysis

Total mercury concentration ([THg]) was determined using a Direct Mercury Analyzer (DMA-80, Milestone Inc, Milestone, Shelton, Connecticut, USA) which combines sample decomposition, catalysis, and atomic absorption spectrophotometry (Harley et al. 2015). The detection level was set to the lowest point on the calibration curve as 0.5 ng. Standard reference materials (SRMs) were selected in order to approximate both expected [THg] as well as simulate the wide variety of matrices analyzed in this study. SRMs used were IAEA 085 and IAEA 086 (hair matrix, International Atomic Energy Agency, Vienna, Austria), DORM-4 (fish protein, National Research Council of Canada, Ottawa, Canada), and NIST-1570a (spinach, National Institute of Standards and Technology (NRC), Gaithersburg, Maryland, USA) and TORT-3 (lobster hepatopancreas, NRC). An internal laboratory standard (Hg in 3.7% HCl) was also used. Recovery of SRMs for THg were between 87 and 103%. Samples were run in triplicate, except in the event of greater than 15% relative standard deviation (RSD) in which case they were run in quintuplicate. Samples where two replicates were below the detection level of the machine (0.5ng) were only run in duplicate. Replicates were averaged using arithmetic mean prior to use in reporting and statistics. Samples that were BDL were factored in to mean calculations (unless otherwise noted) using a ¼ minimum detection limit (MDL) correction.

4.2.5 Methylmercury analysis

Approximately 0.001-0.05g of sample was digested using two different methods. Nitric acid (30% v/v) was used in hair digestion as recommended by Brooks Rand Inc. Briefly, subsamples were weighed out into 40 mL TraceClean vials (VWR, Radnor PA) and 10mL of 30% HNO_3 was added.

Samples were digested in a hot water bath at 65°C for 36-48 hours. Samples were removed from the water bath and 20mL of milliQ water was added to bring the total digestion volume to 30mL. For a detailed description of the nitric acid digestion see chapter 6 of this dissertation.

For all other sample digestions we followed the established method outlined in Bloom (1989) using potassium hydroxide (KOH) dissolved in methanol (25%). Samples were weighed out in TraceClean vials and 10mL of KOH in methanol was added. Samples were digested in the dark at room temperature for approximately 48 hours, at which point 20mL of methanol was added to bring the total digestion volume to 30mL. All digestions were analyzed within two weeks.

Aliquots of samples and SRMs (liquid standard, IAEA-085 and IAEA-086) were run on the Brooks Rand methylmercury cold vapor atomic fluorescence spectroscopy (CVAFS) detection system (including the MERX autosampler) using a 7 point calibration curve Taylor et al. (2011). Methylmercury was analyzed using the Brooks Rand Model III detector (Brooks Rand, Seattle, Washington, USA) using a modified method of EPA Method 1630 in order to incorporate the MERX autosampler. Briefly, 40mL glass vials were filled with milliQ water, 300 µL of acetate buffer, and a 30-100µL aliquot of digestion. An ethylating reagent consisting of 2% sodium tetraethylborate ($NaBEt_4$) in 2%KOH/water was added and the septa caps were screwed on tightly to the vials. Each vial was then sequentially pulled into a bubbler, after which point we followed EPA Method 1630 as outlined in (Harley et al. 2015). Recovery of SRMs for MeHg was 95.8 ± 7.6%.

Similar to THg, samples were run in triplicate, except in the event of greater than 15% RSD. Samples where two replicates were below the detection level of the machine (0.5pg) were only run in duplicate. Replicates were averaged using arithmetic mean prior to use in reporting and statistics. Concentrations of Hg species are reported in wet weight unless otherwise noted. For fish tissues and tortilla samples this represents the concentration in dry weight which was back-calculated to reflect the concentration of the tissue as to represent the fish purchased by the consumer (without freeze drying).

However, for staple foods however the wet weight will refer to the uncooked food (e.g. dried beans, rice). This should be noted as the concentration of cooked staple foods at the "end of the fork" (i.e. as it reaches the consumer) is likely to be lower than what is reported here since the foods can take on water during cooking and are often mixed with other ingredients.

4.2.6 Stable isotope analysis

Stable isotopes of carbon and nitrogen were analyzed similar to Bentzen et al. (2014). Approximately 0.2-0.5mg of dry sample was folded into pressed tin capsules (Elemental Microanalysis, Cambridge, UK) and analyzed using a DeltaVPlus continuous-flow isotopic ratio mass spectrometer (CFIRMS, Thermo Scientific, Bremen, Germany) and elemental analyzer (Costech Scientific, Valencia, CA, USA) at the Alaska Stable Isotope Facility (ASIF) at UAF. Standard reference materials (meat based protein peptone, Sigma Chemical, St. Louis, Missouri, USA) were analyzed approximately every 10 samples to ensure ongoing precision, and blank samples were analyzed approximately every 20 samples to detect drifting values or contamination. Recovery of the peptone stable isotope SRM was 101 ± 2.1%.

Stable isotope values are presented in δ notation that is expressed as a part per thousand (‰) deviation from internationally recognized standards (carbon – Vienna PeeDee belemnite, nitrogen-atmospheric air). Thus,

Equation 4.1-4.2

$$\delta N^{15}\ (‰) = \left\{ \frac{\left(\frac{sample\ N^{15}}{sample\ N^{14}}\right) - \left(\frac{standard\ N^{15}}{standard\ N^{14}}\right)}{\left(\frac{standard\ N^{15}}{standard\ N^{14}}\right)} \right\} \cdot (1000)$$

and

$$\delta C^{13}\ (‰) = \left\{ \frac{\left(\frac{sample\ C^{13}}{sample\ C^{12}}\right) - \left(\frac{standard\ C^{13}}{standard\ C^{12}}\right)}{\left(\frac{standard\ C^{13}}{standard\ C^{12}}\right)} \right\} \cdot (1000\ )$$

Samples were run in two or more replicates. In cases where the RSD for values exceeded 20%, samples were rerun in triplicate or higher. The mean of replicates was obtained for each sample and used in further analyses.

4.2.7 Stable isotope mixing model (SIMM)

In order to conduct a non-biased quantitative assessment of the potential contributions of different items in the participants diet we employed a mixing model based on a Bayesian framework ('simmr' in R, (Parnell et al. 2010). We followed the best practices for SIMM as outlined in Phillips et al. (2014). In addition to the traditional Bayesian two-dimensional mixing model approach, this method allows corrections for the relative concentrations of C and N in our source data. Although not always recommended, this correction has been suggested in instances where there are large differences in elemental concentrations, as is sometimes found in omnivorous diets (Koch and Phillips 2002, Phillips et al. 2014). Since we sampled both staple foods (relatively low N concentrations) as well as seafood (relatively high N concentrations), we had large variation in elemental concentrations of our sources (Figure 4.C-1); we therefore employed the concentration dependent correction to our model. We also employed a k-neighbor joining algorithm (Rosing et al. 1998) to group sources according to similarity in δ^{15}N and δ^{13}C.

Mixing models are dependent on a trophic enrichment factor (TEF) defined as the difference between the stable isotope ratios between the diet items and the tissue of interest in the consumer. These are defined using Δ notation as

Equations 4.3-4.4

$$\Delta \mathrm{N}^{15} = \delta N^{15}{}_{tissue} - \delta N^{15}{}_{diet}$$

$$\Delta \mathrm{C}^{13} = \delta C^{13}{}_{tissue} - \delta C^{13}{}_{diet}$$

Diet items need to be adjusted based on the TEF in order to accurately assess the contribution of each item to the consumer's diet. It has been suggested that the TEF is the most important factor in SIMMs, and small variations in TEF values can lead to large differences in assessments (Martínez del Rio et al. 2009, Caut et al. 2009). Values based on experimental controlled feeding studies have indicated that TEFs are species, tissue, sex, and diet specific (Roth and Hobson 2000, Newsome et al. 2010, Holá et al. 2015). Unfortunately, there are few experimental studies examining TEFs for human hair, and even fewer that have examined females specifically. One study examined $\Delta^{15}N$ and $\Delta^{13}C$ in women (n=20) from Fiji with a similar diet to the women in our study (fish, staples, meat, shellfish, Hedges et al. 2009). TEFs for nitrogen in the literature are somewhat variable, but the convention has been a 3-4‰ increase per trophic level in $\delta^{15}N$, although most studies examining hair or keratin have found this value to be slightly higher (Hedges et al. 2009, Holá et al. 2015). In this study we use TEFs as provided in Caut et al. (2009) for mammalian hair, where $\Delta^{15}N=2.59$ and $\Delta^{13}C=-0.417(\delta^{13}C_{source})-7.878$.

We utilized the *simmr* package (Parnell et al. 2010) developed for the R programming language (R Core Team 2017) using 100,000 iterations and burn-in runs. Using these values, we were able to achieve good convergence based on the Gelmen diagnostics. All further statistical analysis was done in R (R Core Team 2017). All graphical representations were generated using the ggplot2 package (Wickham 2009).

4.4 Results

4.4.1 Mercury and methylmercury in diet items

4.4.1.1 Fish and seafood

THg and $MeHg^+$ concentrations in green crabs and chocolate clams were typically low, as presented in Table 4.1. Mean [THg] were lowest in invertebrate species (< 3 ppb in chocolate clam, < 30 ppb in green crab) and highest in mojarra (*Diapterus peruvianus*, 202 ppb, see vignette). Methylmercury concentrations were highly correlated to [THg] and the average $\%MeHg^+$ of THg was determined as the

slope parameter (a) using a robust linear regression where $[MeHg^+] = a \bullet [THg] + b$ as described in (Wagemann et al. 1997). This method has been shown to be a robust estimator to calculate average ratios of highly correlated variables. This value for all fish and seafood samples was 0.94 (95% confidence interval 0.931-0.943), indicating that the average $\%MeHg^+$ of THg was 94% for all marine samples. Standard errors for crab species were elevated by one carapace and one claw sample that had concentrations > 100 ppb for both THg and $MeHg^+$. The $\%MeHg^+$ for these samples were 94% and 89%, respectively.

4.4.1.2 Rice and other staples

Of the bean and rice samples that were donated from our study participants, no bean samples (0/70, 0%) had concentrations above minimum detection level for $MeHg^+$, while nearly all rice samples (65/70, 93%) had detectable $[MeHg^+]$. The geometric mean (± standard error (SE)) of $[MeHg^+]$ in rice was 2.16 ± 0.09 ppb ww, with the minimum concentration BDL and the highest concentration of 5.17 ppb ww.

No bean or rice samples had concentrations of THg above the detection limit for this assay that was approximately16 ppb. We also tested store bought items including 10 flour samples (5 corn and 5 wheat) and 27 tortillas (15 flour and 12 flour). None of the tortilla or flour samples had consistently detectable concentrations of $MeHg^+$.

Percent $MeHg^+$ was not calculated for staple foods due to many samples being below the detection limit for [THg] and the disparate detection levels of the respective assays. Our minimum detection level (MDL) for [THg] was approximately 16 ppb, while the MDL for $[MeHg^+]$ was at least an order of magnitude less than this (approximately 0.5 ppb). Thus, some samples had detectable concentrations of $[MeHg^+]$ but concentrations of [THg] that were below detection level (BDL).

4.4.1.3 Human hair mercury and methylmercury values

Concentrations of Hg in hair samples are presented in Table 4.2. Geometric mean (±SE) of [THg] was 658 ± 74 and [$MeHg^+$] was 395 ± 56 ppb ww. There were 17 individuals with [THg] above the EPA reference concentration of 1 ppm (Rice et al. 2003, Trasande et al. 2016). Average percent $MeHg^+$, derived using robust linear regression as described above, indicated that on average 76% of the [THg] in hair was $MeHg^+$ (R^2=0.74), although 17 individuals had %$MeHg^+$ less than 50% and 3 individuals had %$MeHg^+$ less than 25%. The [THg] was significantly lower in this study than in Gaxiola-Robles et al. (2014) when compared using a student's t-test (p=0.001).

4.4.1.4 Stable isotope values for human hair

Stable isotopes of carbon ($\delta^{13}C$) ranged from -15.22 to -18.31 (median= -17.03, standard error=0.06) and nitrogen ($\delta^{15}N$) ranged from 8.39 to 10.76 (median 9.24, SE=0.06). The $\delta^{13}C$ and $\delta^{15}N$ were not well correlated (r = 0.18). There was a significant positive association between [THg] and $\delta^{15}N$ ($p<<0.01$, R^2=0.23) as well as [MeHg] and $\delta^{15}N$ ($p<<0.01$, R^2=0.37), although considerably more variability in [$MeHg^+$] was explained by $\delta^{15}N$. Percent $MeHg^+$ was also positively associated with $\delta^{15}N$ ($p<<0.01$, R^2=0.12, Figure 4.2) although not $\delta^{13}C$.

4.4.2 Questionnaire Responses

Neither $MeHg^+$ nor THg varied significantly between groups of self-reported fish consumption (Figure 4.3a). We also noted no significant differences between fish consumption groups and $\delta^{15}N$ values (Figure 4.3b). This was the case for groups based on the frequency of consumption of seafood (not shown).

There was a significant difference in percent $MeHg^+$ between participants who had zero dental amalgams and participants who had one or more (students t-test, p=0.02, Figure 4.4). Due to homogeneity of responses, we did not make comparisons of Hg species related to smoking.

4.4.3 Stable isotope mixing models

We determined $\delta^{15}N$ and $\delta^{13}C$ values from each finfish, seafood, and staple food sample analyzed and executed a mixing model to attempt to account for the relative contribution of each diet item in the source. Our initial model contained each fish species (i.e. Table 4.1) and staple food (rice, corn, beans, etc.) as a separate source. However, this model was inhibited by too many sources due to overlapping stable isotope ranges of many food items. We employed a k-nearest neighbor algorithm (Rosing et al. 1998) to group sources according to similarity in $\delta^{15}N$ and $\delta^{13}C$ (Figure 4.5) and we identified six discrete groups which were fed back into the SIMM. We restricted our model to six sources rather than further grouping because smaller groups, while potentially mathematically sound, would differ largely from groupings based on ecological and dietary considerations (for instance, mojarra would be grouped with crabs and clams). The $\delta^{15}N$ and $\delta^{13}C$ values (±sd) for each diet group (as well as values from the literature for beef and chicken) are presented in Figure 4.6.

The results of the SIMM output are displayed in Figure 4.7. The percent of diet attributed to mojarra (mean proportion = 0.01, standard deviation(SD)=0.01) and fish (mean proportion = 0.02, SD=0.01) were quite low, while beans+flour and rice make up significant, although somewhat variable proportions of diet. Corn made up a very significant proportion of all participants diet (mean proportion = 0.71, SD=0.01). Mean proportions and standard deviation for each group are presented in Table 4.3.

In order to determine if our chemical analysis correlated with self-reported consumption habits, we reran these mixing models separating groups according to their frequency of seafood consumption. To simplify this analysis, we refined the groups into three categories (1) once per month or less, (2) once every other week, and (3) once per week or more. By rerunning the model, we found that there were only small differences in the estimated portions of fish in the diet of each of the three groups (Figure 4.7a). We then ranked these three groups according to model output and found that the most frequent consumption group (at least once per week) was selected as the highest fish consumers in 39% of iterations, as compared to 31% and 30% for the other two groups.

We also ran our SIMM to compare fish consumption across groups generated according to their $MeHg^+$ concentration and $\%MeHg^+$ in hair (Figure 4.7b). Using the three approximately equal sized groupings of participants, we found that model selected the high $[MeHg^+]$ group as the highest fish consumers approximately 50% of the time. The model selected the high $\%MeHg^+$ group 43% of iterations (Figure 4.7c).

4.5 Discussion

Hair mercury concentrations in this study were lower than in Gaxiola-Robles et al. (2014), and an order of magnitude lower than concentrations in pregnant women from known high consumption communities (i.e. the Seychelles, van Wijngaarden et al. 2017). Concentrations of THg reported here are less than the 95 percentile of frequent fish consumers reported in the NHANES study (McDowell et al. 2004), although nearly all (65 out of 70, 96%) of the individuals in this study had hair [THg] higher than the median concentration of all participants in that study.

There are several potential reasons for the different average [THg] hair concentrations between the two studies. Although these were not the same women examined in the previous study (Gaxiola-Robles et al. 2014), they were from a similar area, age, and socio-economic class. One explanation could be a general decrease in fish consumption, although our $\delta^{15}N$ did not differ significantly from those of Bentzen et al. (2014). However, a switch in preferred species from one with higher [THg] (i.e. mojarra) to one with lower [THg] (i.e. cochito) could explain differences in [THg] that were not associated with changes in $\delta^{15}N$. Although species specific consumption was not assessed in the previous studies, in our study a large portion of respondents reported consuming pierna (41%) and cochito (30%), two species that we found to have relatively low [THg].

Hair $[MeHg^+]$ is not commonly measured, and is generally assumed that the percent $MeHg^+$ of total Hg in hair is at least 80% (Cernichiari et al. 1995). However, to our knowledge there is no standardized method of calculating average $\%MeHg^+$, with some reports using simple arithmetic means

(Harada et al. 1998), while others have advocated for a robust linear regression (Wagemann et al. 1997) making it difficult to compare results across studies. The median $\%MeHg^{+}$ in our study was 67%, although 17 individuals had $\%MeHg^{+}$ less than 50%. While we support the calculation of the average ratio of $MeHg^{+}$:THg using methods other than arithmetic mean, we suggest here that there were individuals whose $\%MeHg^{+}$ values differ greatly from the slope calculated parameter. Different tissues can have different $\%MeHg^{+}$ (Squadrone et al. 2015) related to the cysteine content, lipophilicity, or *in situ* demethylation, thus it is conceivable that hair $\%MeHg^{+}$ could vary from blood or urine. An alternative explanation is that these individuals have exposure to sources of inorganic Hg in addition to $MeHg^{+}$ through diet.

Sources of inorganic mercury exposure that have been implicated for Mexican citizens include cosmetic and beauty supplies, some of which contain mercury concentrations between 20,000 and 36,000 ppm (Dickenson et al. 2013). Imported cosmetic products were identified as a potential source of inorganic mercury exposure in adults from New York City (McKelvey et al. 2011). It has been suggested that inorganic mercury is absorbed through skin across the epidermis via sweat glands and sebaceous glands (Chan 2011, Park and Zheng 2012), which would explain high concentrations of Hg found in urine of patients displaying symptoms of Hg toxicity after self-reporting using a Mexican skin lightening cream (Weldon et al. 2000). Indeed, a study in 2011 found that 6 of 15 creams produced in Mexico had detectable concentrations of Hg, some as high as 36,000 ppm (Peregrino et al. 2011), well above the FDA's allowable limit of 1 ppm. Although the dermal absorption rate of the Hg from lightening creams is not well described, there is strong evidence that there is at least partial absorption (Clarkson and Magos 2006, Peregrino et al. 2011) and even poor absorption efficiencies could lead to potentially toxic exposures.

Concentrations of THg and $MeHg^{+}$ in the chocolate clam were quite low (2-5ppb), which is consistent with the current concentration reported by the FDA and Atwell et al. (1998). To our knowledge, this is the first reporting of Hg concentrations in this species from this region. Concentrations

of THg and $MeHg^+$ were slightly higher in the crab samples, and although Hg concentrations are not commonly measured in crab species our values were more or less consistent with previous reports (Burger et al. 2007). One crab sample of both carapace and claw muscle tissue had concentrations of THg and $MeHg^+$ 5-10 times higher than the other samples (135 [$MeHg^+$] and 112ppb [THg] ww). Interestingly, this individual had the lowest $\delta^{15}N$ and $\delta^{13}C$ values of the crabs, nearly a full trophic level (3‰) lower than the mean $\delta^{15}N$. Crabs and other detritivores are likely exposed to variable concentrations of Hg due to opportunistic feeding on detritus, so variation in [Hg] and stable isotopes are perhaps not unexpected. While it is surprising to see significantly higher concentrations in an individual feeding on a lower trophic level, we will not draw any conclusions from one sample on a poorly studied species, although further analysis of [Hg] in detritivores from the region are warranted.

Concentrations of Hg in finfish were generally low, and based on their stable isotope profiles most appeared to be low trophic level feeders. However, even the higher trophic level species (cabrilla *Mycteroperca rosacea* and sierra *Scomberomorus sierra*, mean $\delta^{15}N > 18$) had geometric mean concentrations less than 100 ppb. We caution that despite the low concentrations reported here, there could be seasonal or spatial variation in Hg concentrations within a species. More robust sample sizes over larger temporal and spatial settings should be assessed prior to considering consumption recommendations. There were a few species that were reported to be consumed by the study participants that were not measured for Hg including shrimp and scallops, however these items are generally not known to have high [Hg]. Some studies from this region have shown high [THg] in sharks and elasmobranchs (Barrera-García et al. 2012). These species were not asked about specifically, but were not mentioned in self-reporting.

Stable isotopes from hair samples were analyzed in order to assess potential sources of Hg through diet (Bentzen et al. 2014). Overall, our isotope values were very similar to Bentzen et al. (2014), although in this study we did not observe nearly as broad a range in [THg]. Interestingly, $\%MeHg^+$ appeared to be positively associated with $\delta^{15}N$. Bentzen et al. (2014) found that $\delta^{15}N$ was associated with

[THg] that was likely driven by finfish and seafood consumption. We also found a positive association between $\delta^{15}N$ and [THg], and a stronger association between $\delta^{15}N$ and [$MeHg^+$] and $\%MeHg^+$. This supports the notion that the seafood and fish are sources of $MeHg^+$ exposure, however the low $\%MeHg^+$ among infrequent fish consumers ($\delta^{15}N < 9$) could also potentially be explained by these individuals having exposure to Hg (potentially inorganic) via other routes than seafood. Dental amalgams have also been known to increase tissular inorganic Hg concentrations, and other studies have found that some of the variation in $\%MeHg^+$ can be explained by the presence of dental amalgams (Vieira et al. 2013). In our study, $\%MeHg^+$ was indeed lower in individuals with one or more dental amalgams, although there were no significant differences in [THg] or [$MeHg^+$] between those with amalgams and those without. There are numerous factors to consider regarding Hg exposure via dental amalgams including the timing of the procedure as well as the type of amalgam used. It would be worthwhile to design a specific study examining Hg exposure via dental amalgams in this population.

Corn has a fairly unique $\delta^{13}C$ signature among human staple foods due to its C_4 carbon fixation which results in less depletion of the heavier isotope (O'Brien 2015). It should be noted that, while all respondents had significant contribution of corn in their diets, this does not imply that only corn products (i.e. flour) are contributing. Many livestock species including cattle and chicken are fed diets with large proportion of corn, thus their $\delta^{13}C$ signatures are quite similar to corn (Jahren and Kraft 2008). We did not measure stable isotopes in these sources, however many of the study participants reported frequent consumption of both beef, chicken, and pork, which would contribute to the corn-like $\delta^{13}C$ we observe here (see Figure 4.6).

There are a number of assumptions and sources of error built into SIMMs, and while we attempted to control for most of these there are a few confounding factors that should be addressed. First, there is evidence that individual variation in uptake and metabolism can lead to variation in stable isotope values in consumers. For instance (Sponheimer et al. 2003), found large variation (up to 3‰) in $\delta^{15}N$ values in mammalian hair that were fed identical diets. It is also possible that individuals in this study

could have exogenously altered the chemical composition of their hair, i.e. via bleaching or washing. Indeed, a number of samples had evidence of artificial coloration. However, it appears that stable isotope values of human hair are unaffected by shampoo and only slightly affected by aggressive bleaching (O'Connell and Hedges 1999), thus we don't see this as a large source of error.

Overall, our SIMM agreed with self-reported fish consumption. We did not see significantly different $\delta^{15}N$ values across fish consumption groups, however using a stable isotope mixing model we found that frequent fish consumers had slightly higher proportions of fish in their diet. Our model comparing groups based on $MeHg^{+}$ concentrations and $\%MeHg^{+}$ in hair was more successful at distinguishing the chemical signature of fish consumption, which provides further evidence that chemical analysis of diet is warranted even in circumstances where dietary recall is possible.

4.6 Vignette – The Curious Case of Mojarra

Mojarra is the common name of fish of the family Gerreidae, although in Latin America the term mojarra is used for members of the cichlid family (Cichlidae). In Mexico, farmed mojarra (also known as tilapia) accounts for 95% of national production (CONAPESCA 2013), and since its inception Mexico has become one the largest producers of farmed mojarra in the world (Fitzsimmons 2000, CONAPESCA 2013). Both farmed mojarra (*Tilapia spp.*) and wild mojarra (*Diapterus spp.*) are consumed by residents of Mexico, although tilapia is generally known as mojarra de agua dulce (freshwater mojarra). Farmed freshwater tilapia is generally regarded as low in mercury (EPA lists their average concentration at 13 ppb wet weight), and are in fact recommended as a "Best Choice" for consumption (2-3 times per week) for pregnant women by the US FDA and US EPA. The *D. peruvianus* on the other hand has been noted to have mean concentrations of 580 ppb ww from the Sinaloa coast of Mexico, a value which is higher than the general guidance level for fish mercury from both the World Health Organization (WHO) and the European Commission (EC) (Ruelas-Inzunza et al. 2008). Another study found mean [THg] in *D. peruvianus* to be the highest (2,556 ppb dry weight) of 19 species analyzed from the Gulf of California (García-Hernández et al. 2007).

[THg] found in *D. peruvianus* are slightly unusual considering the size and ecology of the species. The mean length of *D. peruvianus* in this study (16.7 cm, n=12) was in agreement with previous reports on the species (16.8 cm, Spanopoulos-Zarco et al. 2015), yet it was notably smaller than other fish species assessed in this study. There are only a few resources regarding dietary assessments for the species, however most indicate that they are benthic carnivorous or omnivorous fish whose prey include mainly bivalves and marine worms (Phyla Mollusca and Echinodermata), although Lopez-Peralta and Arcila (Lopez-Peralta and Arcila 2002) also note detritus as a dietary component in their stomach content analysis. Further investigations into the feeding ecology, life history, and metabolism of this species might be warranted to assess their role in human Hg exposure.

4.7 Conclusion

We have presented here [THg] and [$MeHg^+$] in commonly consumed fish species from La Paz, Baja California, Mexico. To our knowledge, this is the first reporting of Hg in some of these species from this region. We found lower Hg concentrations in hair samples from pregnant women than previous reports (Gaxiola-Robles et al. 2014), which could indicate a change in dietary preference. Further monitoring of human Hg exposure and Hg dynamics in the local environment could provide a more complete picture of Hg exposure for humans in this area.

4.8 Acknowledgements

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4.9 Figures

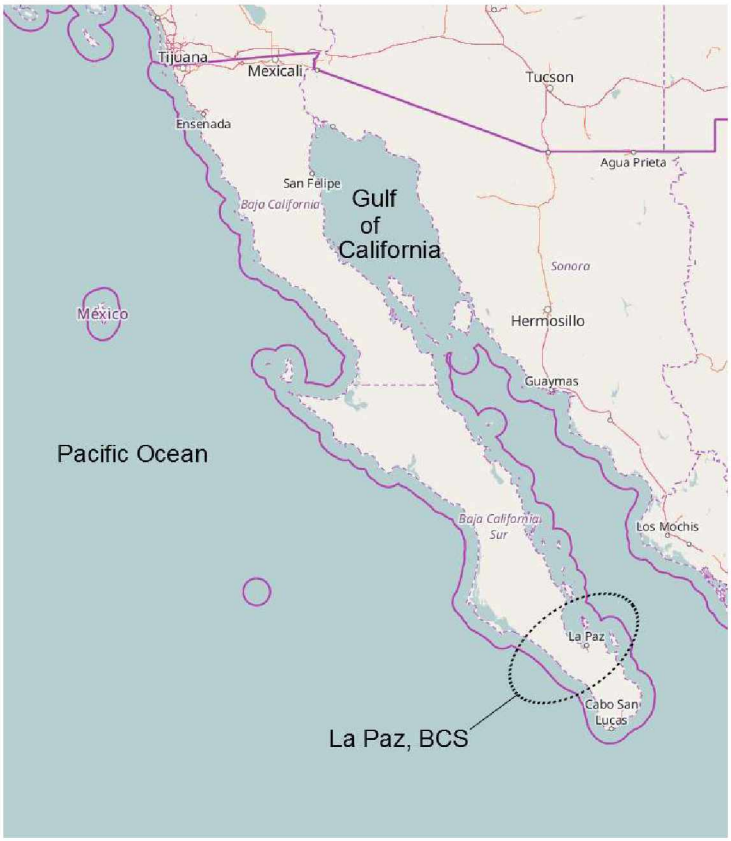

Figure 4.1 – A map of Baja California. La Paz, Baja California Sur (BCS) is situated on the Gulf of California, although it lies close to the Pacific Ocean near the fishing villages of Todos Santos and El Pescadero. Commercial fish are harvested from both coasts of the peninsula. The purple line approximately denotes the 12-mile territorial zone. Map is from OpenStreetMaps.

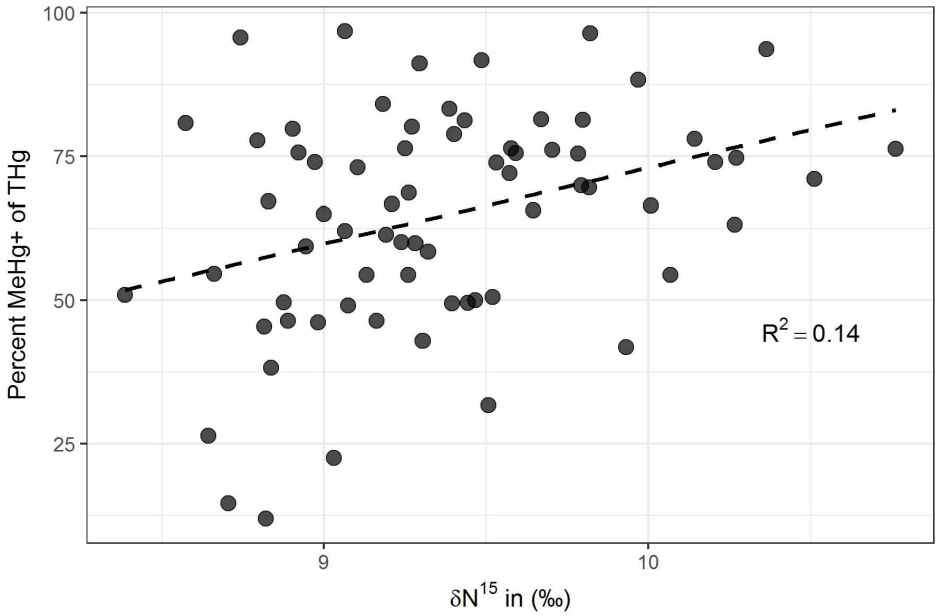

Figure 4.2 – Linear relationship between %$MeHg^{+}$ of THg iand $\delta^{15}N$, an estimator of fish consumption, in human hair samples (n=70). Seventeen individuals had %$MeHg^{+}$ less than 50%.

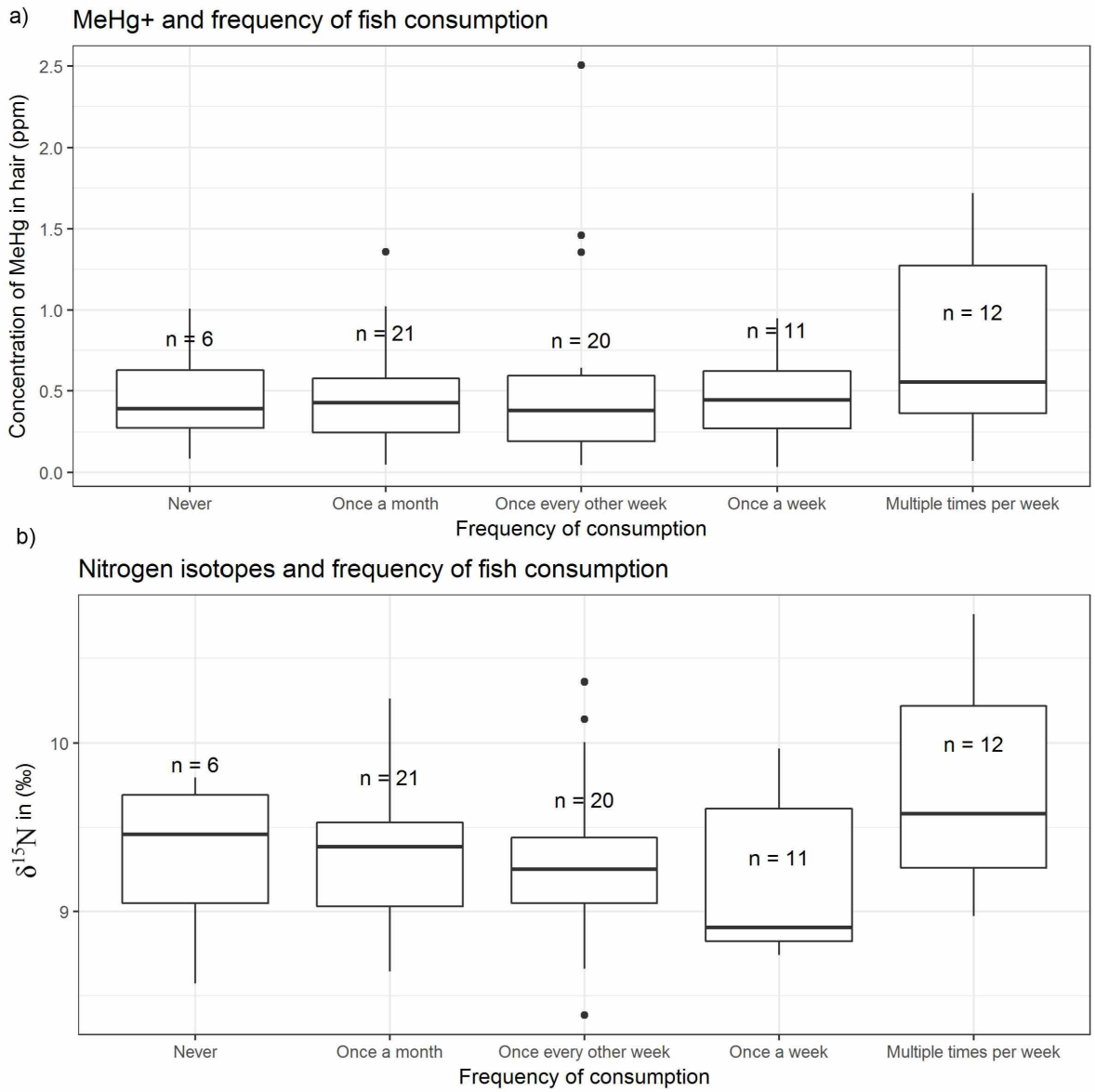

Figure 4.3 – (a) Concentrations of $MeHg^+$ in hair samples grouped by self-reported fish consumption, and (b) $\delta^{15}N$ in hair based on self-reported consumption of fish. Boxplots represent first quartile, median, and third quartile while whiskers represent the highest and lowest datum within 1.5 interquartile range (IQR). Individual points are data outside 1.5*IQR.

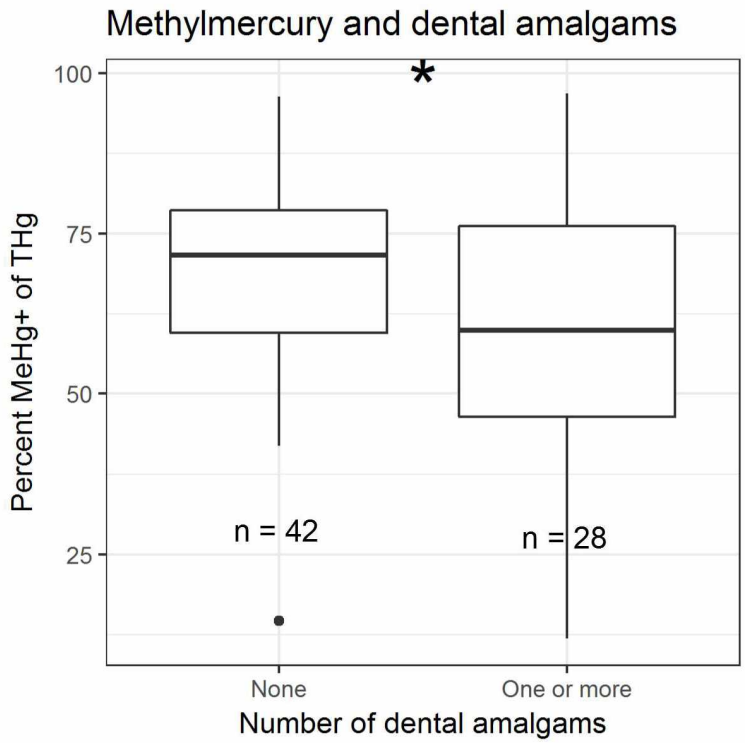

Figure 4.4 – Differences in the percent $MeHg^{+}$ of THg between study participants with and without dental amalgams. Asterisk indicates significance at the α=0.05 level (student's t-test).

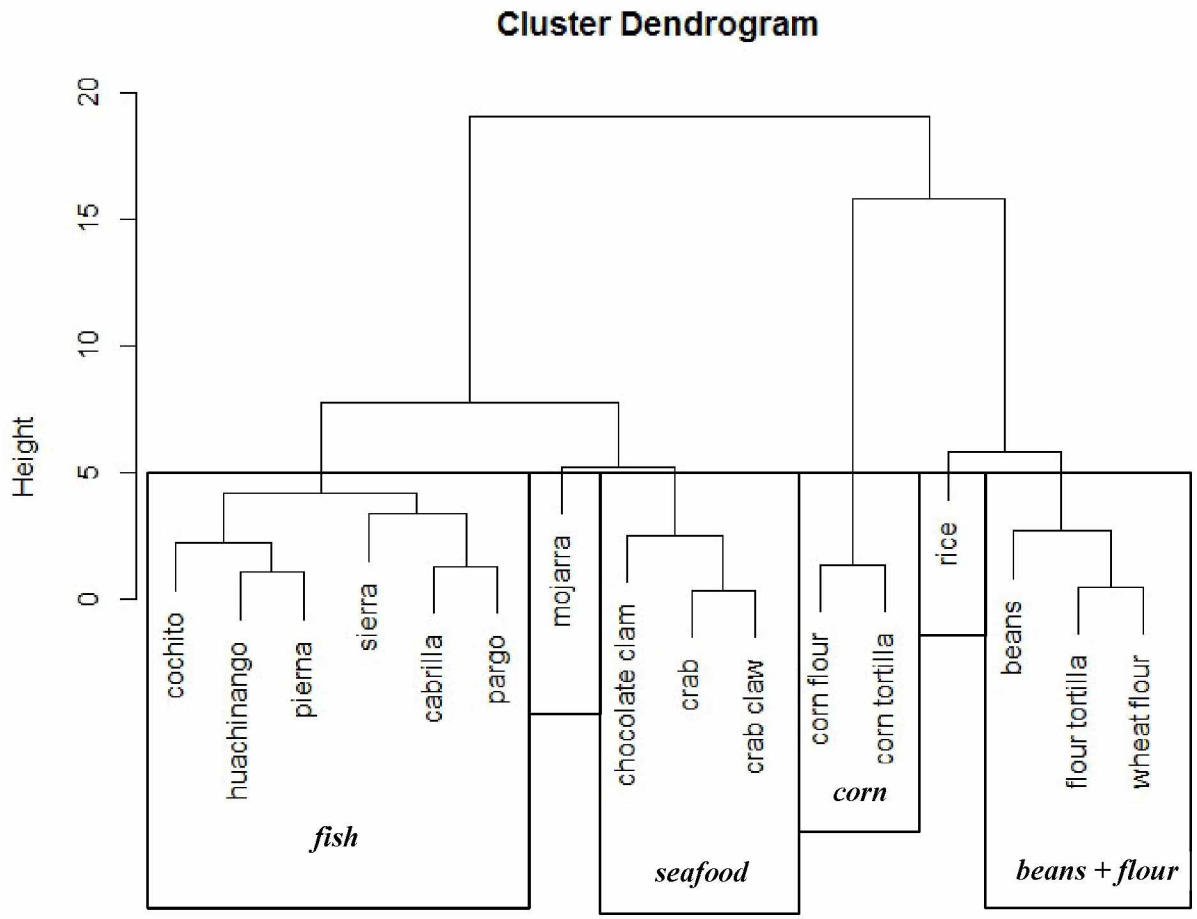

Figure 4.5 – Results of clustering k-neighbor joining clustering algorithm based on $\delta^{15}N$ and $\delta^{13}C$. Groupings (n=6) used in further analysis are shown in boxes.

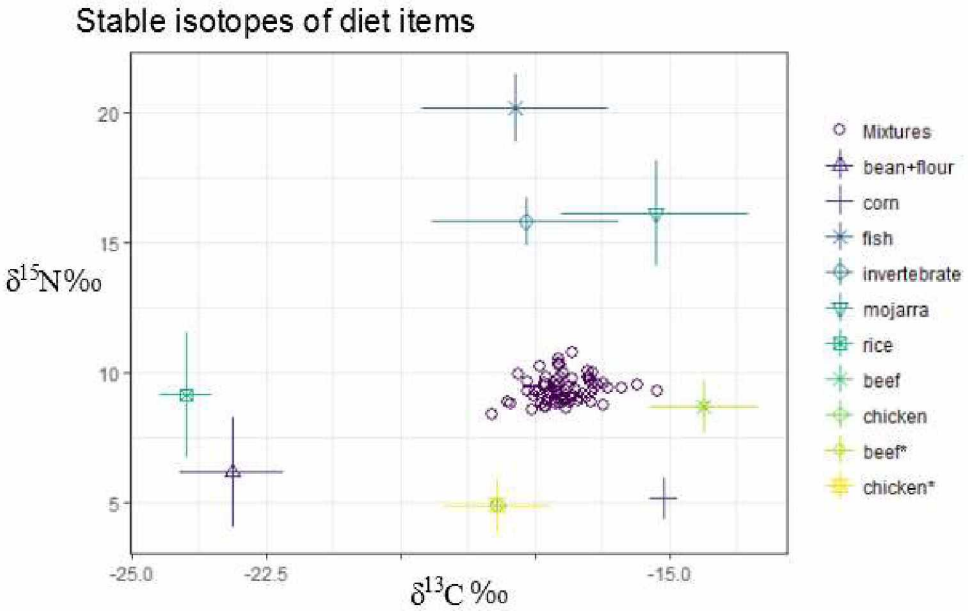

Figure 4.6 – Average stable isotope values of grouped dietary items (grouped according to k-neighbor clusters presented in Figure 4.5. Values for beef and chicken are provided from Jarhen and Kraft (2008) in order to show the corn signature present in these protein souces. Mixtures provided are individual hair samples (n=70) from pregnant women in La Paz, Baja California, Mexico.

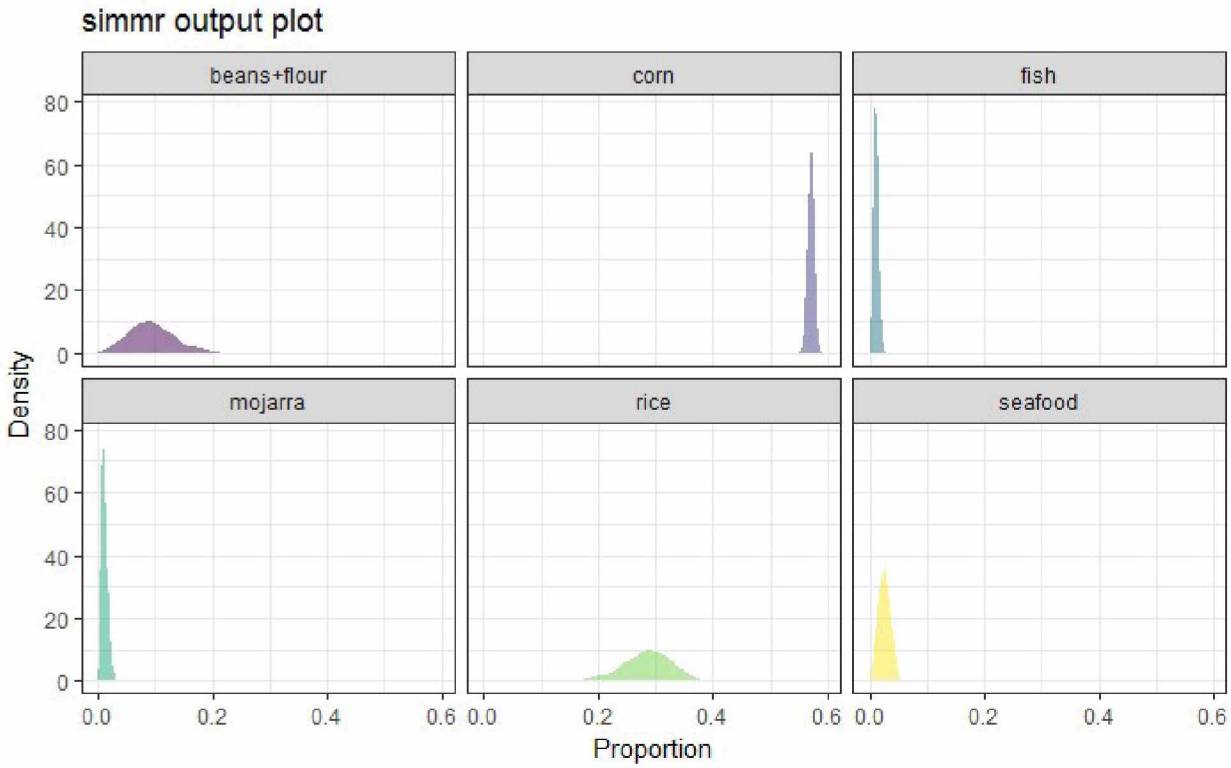

Figure 4.7 – Proportions of each diet group for all study participants (n=70) based on the results of the stable isotope mixing model (SIMM) following source clustering. The fish cluster contains cabrilla, cochito, huachinango, pargo, pierna and sierra. The seafood cluster contains both green crab and chocolate clam.

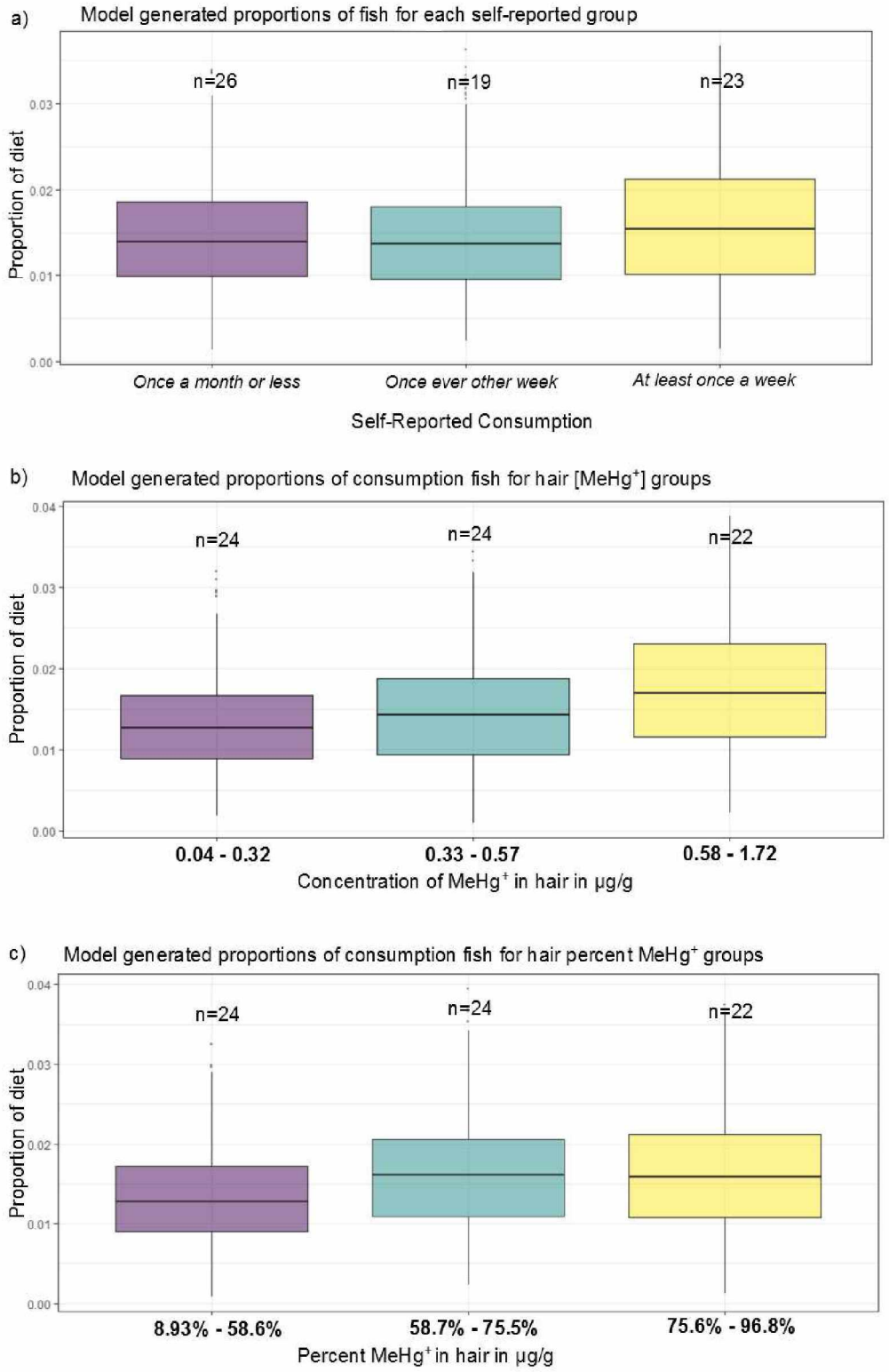

Figure 4.8 – a) *Simmr* model output proportions of fish in the diet of each self-reported fish consumption group, b) model output proportions of fish consumption based on groups according to [$MeHg^+$] in hair, and c) model output proportions for groups based on %$MeHg^+$ in hair.

4.10 Tables

Table 4.1 – Mercury concentrations for fish and seafood. Data are presented as geometric mean ± standard error. Percent $MeHg^+$ for each species is calculated using geometric mean of individually calculated $[MeHg^+]/[THg]$.

Common name		Species	Tissue	Number	[THg] in ppb ww	$[MeHg^+]$ in ppb ww	%MeHg
Spanish	English						
almejas chocolates	chocolate clam	*Megapitaria squalida*	mantle	5	2.37 ± 0.05	1.40 ± 0.04	59%
			abductor	5	2.91 ± 0.21	2.37 ± 0.22	82%
			foot	5	2.96 ± 0.17	2.24 ± 0.15	75%
cangrejo verde	green crab	*Callinectes bellicosus*	carapace	6	27.50 ± 21.00	25.63 ± 20.30	93%
			claw	8	20.62 ± 12.11	19.30 ± 10.75	94%
cabrilla	leopard grouper	*Mycteroperca rosacea*	muscle	4	86.80 ± 18.80	78.90 ± 19.22	91%
cochito	finescale triggerfish	*Balister polylepis*	muscle	4	23.00 ± 20.56	21.90 ± 20.87	95%
huachinanago	red snapper	*Lutjanus peru*	muscle	4	30.09 ± 2.89	29.44 ± 2.98	98%
mojarra	Peruvian mojarra	*Diapterus peruvianus*	muscle	8	196.68 ± 36.04	178.54 ± 30.74	91%
pargo	yellow snapper	*Lutjanus argentiventris*	muscle	5	77.64 ± 13.91	71.48 ± 14.16	92%
pierna	ocean whitefish	*Caulolatilus princeps*	muscle	6	51.57 ± 26.76	46.76 ± 30.57	91%
sierra	Pacific sierra	*Scomberomorus sierra*	muscle	6	73.42 ± 2.57	70.21 ± 4.01	96%

[THg] – concentration of total mercury
$[MeHg^+]$ – concentration of methylmercury
ppb – parts per billion (µg/kg)
ww – wet weight
%MeHg – $[MeHg^+] / [THg]$

Table 4.2 – Mercury concentrations in human hair. Values expressed as geometric mean in parts per billion (μg/kg) wet weight.

Study	Hg Species	Median	Geometric mean	Standard error	n > 1ppm	n > 5ppm
This study	[THg]	688	658 ±	74	17/70 (24%)	0 (0%)
	[$MeHg^+$]	432	395 ±	56	10/70 (14%)	0 (0%)
	%$MeHg^+$*	76.3%	60.4%	2.3%		
Gaxiola-Robles *et al.* (2014)	[THg]	1,520	1,389	463	54/75 (72%)	6/75 (8%)

*Percent $MeHg^+$ calculated as the slope of the regression (a) where [MeHg] = a • [THg] + b. For further explanation see text and Wagemann et al. (1997).

Table 4.3 – Mean proportions and standard deviations (s.d.) of items in the diet of all study individuals generated by the mixing model (*simmr)*.

Food Item	Proportion	s.d.
corn	0.71	0.01
rice	0.15	0.04
bean+flour	0.08	0.04
invertebrates	0.03	0.01
fish	0.02	0.01
mojarra	0.01	0.01

4.11 Appendix C

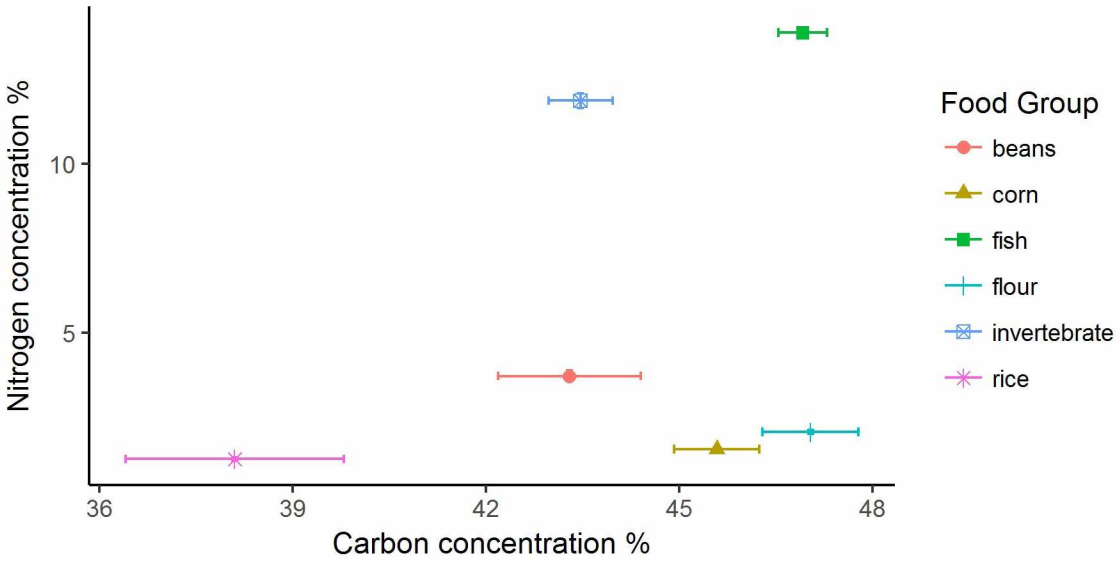

Figure 4.C-1 – Differences in elemental (carbon and nitrogen) concentrations of different foods.

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Chapter 5 – **What does One Health want?**[7]

[7] John Harley

5.1 Where is One Health Going?

One Health has traditionally incorporated numerous scientific disciplines such as veterinary science, human medicine, and environmental studies. As a collaborative cross-disciplinary effort to increase holistic perspectives in health research, One Health is often supportive of and driven by advocacy. To this end, some have argued for the functional incorporation of social sciences under the One Health banner. Stephen (2014) reasons that if the goal of One Health is to illustrate and foster the linkage between animal and human health, public perception of animals and the environment are paramount to these efforts. Examining ways in which humans interact and respond to the natural world is a necessary step in the effective collection and dispersion of knowledge.

A discussion of the nuanced intersection between science public outreach and advocacy is not an imprudent one and certainly relevant here. Scott et al. (2007), among others, points out the inherent dissonance between objective science and advocacy, namely that advocates are less likely, in practice and perception, to be viewed as neutral figures in policy decisions. In the context of One Health, remaining neutral with respect to, say, a devastating emerging infectious disease would not only be impractical but potentially dangerous. However, in other areas of research, the separation between science and advocacy might be more nuanced. For instance, a narrow focused toxicologist might advise against consumption of marine mammal tissues that may have high concentration of persistent organic pollutants. However, without considering the social or traditional aspects of a treasured diet item, or without working toward positive remediation or supplementation, the relationship between the community and advocates might be damaged (Horton et al. 2016). This could lead to poor community participation or adversity toward scientific research, both of which would be contrary to the goals of One Health (Jardine et al. 2013).

5.2 Recommendations for further research

While the concept of animal models for human health is by no means new, we feel that there is significant improvement that can be made regarding their use and scope. Traditionally, biomedical models have been dominated by a few laboratory species with little genetic diversity (inbred strains), but

as we discussed in Chapters 2 and 3, this might be the wrong context in which to examine human toxicant exposures. Domestic pets have been and should continue to be utilized to examine environmentally relevant human contaminant exposure (Dunlap et al. 2007), and we have shown here how unique domestic animal situations (sled dogs) can even provide a robust sample size (Harley et al. 2016).

There have been several criteria laid out as to what makes a good sentinel species (Basu et al. 2007), and a decade ago anyone examining molecular biology in a sentinel species would have likely advocated for a good sentinel species to have an annotated or assembled genome. However, advances in informatics technologies have increased the speed, efficiency, and applications of *de novo* genome and transcriptome assembly (Grabherr et al. 2011). This major technical hurdle has allowed for the expansion of the list of potential sentinel species to include non-model organisms. While many of the guidelines for sentinel selection remain the same, there are no doubt human populations, such as those living in remote areas or having a unique diet, that will benefit from this development.

As stated earlier, One Health is founded on collaboration and outreach, not only among scientists, but among members of the community and stakeholders. Increased collaboration with not only natural scientists, but social scientists and policy-makers is critical for success. One Health efforts can undoubtedly benefit from social science research into effective means of communicating science and incorporating One Health ideas into policy and action. Care should be taken to foster credibility with both community members and resource shareholders in order to promote community collaborations.

Others have pointed out the dangers of pigeonholing One Health into the realm of zoonotic disease detection and communication (Stephen 2014). There is a danger, with One Health toxicology, of falling into the same tendencies, that is, to simply assess concentrations or toxicoses and conduct risk assessment. However, there is an increasingly prevalent call for efforts to be directed toward prevention and preemption rather than interventions (Grabherr et al. 2011, Baum et al. 2017). One Health toxicology can benefit greatly from this direction, and combining this approach with community interactions (social science) and quantitative outcome assessment (i.e. Baum et al. 2017) will ensure the future success of the continually evolving collaboration.

5.3 The future of One Health

It is interesting in the light of the material presented in this dissertation to reflect on the expansion of the One Health collaboration. We have presented several technologies, methods, and perspectives that have received little, if any, attention within the context of One Health. I have argued here that methods ranging from modern Next-Gen sequencing (Chapter 3) to more established techniques such as C and N stable isotopes mixing models (Chapter 4) can be utilized to inform and interpret questions related to One Health questions and issues. Here I admit to a bias towards fish based food webs and Hg exposure. However, the approaches and perspectives are not limited to fish based efforts.

There are, of course, some limitations to the incorporation of new methods, new sentinel species, or new technologies within the context of One Health. While we often rely on sentinel species for assessing human exposure to environmental contaminants, there are differences in form and function between animals and humans which should be considered. Genetic, phenotypic, physiologic, metabolic, and behavioral differences between humans and sentinel species could alter exposure to contaminants as well as metabolism and physiologic responses. From an –omics perspective, the annotation and ontological understanding of DNA and RNA sequences in wildlife is lagging behind our ability to sequence and assemble high-quality genomes and transcriptomes. However, I have shown here the value in considering non-model organisms as sentinel species, and future efforts to increase informatics knowledge will only increase the efficacy of sentinel species in molecular toxicology. The development of bioinformatics tools under One Health will enable the analysis of data sets which previously might have been considered too complex.

As we proceed with science under One Health, it is worth considering in what direction the momentum is heading, and to what degree, if any, the progress should be directed. We have certainly seen in recent years an expansion of One Health research, and although some have pointed out that concepts of One Health have been around for decades or even centuries (Evans and Leighton 2014), there seems to be, at least in the mind of young scientists, a sense of momentum within the One Health community today. Perhaps, as mentioned in the introduction of this dissertation, increasing modes of communication

have allowed collaboration-centered sciences to flourish. Alternatively, perhaps researchers in One Health associated fields feel the momentousness of impending disruptions to the global system such as a changing climate or microbial antibiotic resistance.

5.4 The goal of this dissertation

If One Health is permitted to be advocacy oriented, it is perhaps fitting that the collected works presented here are given the same allowance, beyond simply the product of a graduate student (PhD). The goal of this research (Chapter 2, 3, & 4), as stated in the Introduction, is to expand the toolkit for researchers investigating questions related to One Health. Scientific progress is certainly assisted by an ever-expanding array of methods, technologies, and perspectives. A minor development in one field might lead to an insightful breakthrough in another, therefore the sharing of ideas and technologies across disciplines should be encouraged and strategically supported.

The data and analyses presented here exemplify the ever expanding and evolving scope of One Health toxicology. Allowing future researchers to have more cognitive frameworks and technical tools available to them will certainly improve the efficacy and frequency in which One Health ideas are researched and communicated. As is the case in any single project, the contribution to the collective knowledge in this dissertation at the point of completion is a small drop in an ocean, but it is my sincere hope that this small drop strengthens the current which seeks to ensure and promote the health of animals, humans, and the environment.

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Chapter 6 (Appendix) – **Validation of an acidic digestion method for the determination of methylmercury in hair samples**[8]

[8]Harley, J.R., K. M. Veeder, L.D. Rea, T.M. O'Hara. In prep. Validation of an acidic digestion method for the determination of methylmercury in hair samples. Nature Methods.

6.1 Abstract

Total mercury concentrations ([THg]) in hair are commonly measured in piscivorous mammals in order to assess Hg exposure. It has been estimated that most THg in hair (~80%) is in the form of monomethylmercury ($MeHg^+$) for piscivorous mammals, although in some systems there may be confounding sources such as industrial or occupational exposure to gaseous inorganic Hg (IHg), which may adsorb directly onto hair. Although IHg species can also exhibit toxicity, $MeHg^+$ is a potent neurotoxin and can cause developmental disorders in developing fetuses and is usually the form of most concern to the population. Thus, when assessing hair Hg against various thresholds of concern, direct measures of [$MeHg^+$] and %$MeHg^+$ might be a better estimator of exposure to $MeHg^+$, rather than using THg as a proxy. Here we present validation of a simple and rapid digestion method for assessing [$MeHg^+$] in mammalian hair samples using the MERX automated methylmercury samples (Brooks Rand) in conjunction with cold-vapor atomic fluorescence spectroscopy (Brooks Rand Model III). Using three hair standard reference materials nitric acid digestions performed better at digesting standard reference materials than the standard KOH/methanol digestion (97% recovery vs. 81% recovery). We also compare digestion volumes of nitric acid in order to assess the efficacy of smaller digestions. As proof of principal, we utilize the nitric acid digestion procedure to analyze $MeHg^+$ in the hair of Steller sea lion pups (*Eumetopias jubatus*) from Alaska. Using this method, we found mean %$MeHg^+$ to be 72%, which is similar to what others have found in piscivorous mammals. This method is a valuable technique for the analysis of hair $MeHg^+$ in mammalian species, and we emphasize that the assessment of $MeHg^+$ and %$MeHg^+$ are extremely valuable for toxicological assessments of humans and wildlife.

6.2 Introduction

Hair has long been used as a strategic sampling matrix to examine exposure to some trace elements in humans and wildlife (Mahaffey 2005; Castellini et al. 2012; van Hoomissen et al. 2015). For most mammals hair is a desirable tissue since 1) it is easily accessible and sampling can be relatively non-invasive, 2) hair has a low turnover rate and can reflect chronic exposures (including temporal trends using segmental analyses), and 3) hair tends to concentrate trace elements at higher concentrations than other commonly sampled tissues (i.e. blood, Liberda et al. 2014; van Hoomissen et al. 2015).

Mercury (Hg) in the environment exists in both inorganic (Hg^0, Hg^{1+}, Hg^{2+}) and organic (R-Hg) forms. Organic Hg is largely in the form monomethylmercury (henceforth methylmercury or $MeHg^+$), is generated from inorganic mercury in aquatic environments via iron or sulfate reducing bacteria. This process was thought to occur largely in sediments, however there is evidence to suggest that this process also occurs in the water column attached to particles or biota that fall beneath the mixed layer (Blum et al. 2013). Methylmercury bioaccumulates in fish and other aquatic organisms and thus detectable concentrations of $MeHg^+$ are common in species and individuals with a marine or freshwater diet (Dietz et al. 2013).

Measurement of total mercury (THg) is common for analysis of hair samples due to ease of analysis and low cost per sample, although some researchers have analyzed $MeHg^+$ in human hair in order to assess potential sources of Hg exposure (Kyle and Ghani 1982; Malm et al. 1995; Voegborlo et al. 2010). While conventional thinking suggests that the percent of THg that is $MeHg^+$ is approximately 80% or higher (Mahaffey 2005; Clarkson and Magos 2006), studies that have analyzed the $\%MeHg^+$ in human populations have occasionally found a large amount of variation (reviewed in Barbosa et al. 2001). The cause for the variation in $\%MeHg^+$ in hair is not well understood, although potential explanations include variation in dietary exposure, differences in toxicokinetics, or environmental adsorption. For instance, Manceau et al. (2016) found that the chemical form of Hg in hair samples could be used to assess potential sources of exposure. In Chapter 4 of this thesis, we found that individuals with dental amalgams

(which contain inorganic Hg) had a lower %$MeHg^+$ in hair than individuals with no dental amalgams. Oskarsson et al. (1996) found that breast milk inorganic Hg was correlated with number of dental amalgams, while organic Hg was correlated with recent fish intake in lactacting women.

There is some evidence that gaseous inorganic Hg may adsorb directly onto hair, at least in simulated occupational exposure (Li et al. 2011). While it is unlikely that the majority of a wildlife or human population is regularly exposed to gaseous concentrations of Hg found in mining sites, there is no reason not to think that some Hg exposure could come from ambient air (Cole et al. 2014) or from personal aerosolized consumer products (i.e. cigarettes, Chiba and Masironi 1992). Although exposure to gaseous Hg can certainly be harmful, the determination of THg in hair almost always assumes that the individual is exposed to Hg which is then excreted into the hair – thus the adsorption of gaseous Hg directly onto the hair is a potentially confounding factor. Gaxiola-Robles et al. (2014) found that passive exposure to cigarette smoke explained a significant amount of variation in hair [THg] from pregnant women in La Paz, Baja California Sur, Mexico.

Exposure to gaseous forms of Hg largely consist of inorganic Hg^0 and Hg cations. Therefore, it may be prudent in certain situations to measure concentrations of $MeHg^+$ in hair, rather than THg (which includes IHg). Compared to IHg, $MeHg^+$ is a potent neurotoxin which has caused a number of mass poising events (Gochfeld 2003). While the chemical is often used in laboratory studies to induce toxicity, longitudinal studies examining impacts of chronic $MeHg^+$ exposure have found only limited and inconsistent evidence of adverse effects in fish consumers of the Seychelles (Myers et al. 2003; van Wijngaarden et al. 2013; Myers et al. 2015). Nevertheless, the monitoring of $MeHg^+$ concentrations is a useful tool in establishing both risk factors and potential biomarkers of toxicity in humans and wildlife species. The identification of both [$MeHg^+$] and the %$MeHg^+$ of THg would provide valuable information regarding potential sources of Hg exposure, which would be inaccessible if only [THg] are measured.

Here we provide validation of a nitric acid method for hair digestion for the analysis of [$MeHg^+$] in hair samples. Our results indicate that this method provides superior results as compared to the basic KOH/methanol digestion. We compare various volumes of nitric acid digestion in order to optimize the digestion, and we demonstrate the applicability of this method to measure $MeHg^+$ in hair samples from Steller sea lion pups from the Aleutian Islands, Alaska.

6.3 Method

The following standard reference materials (SRMs) were used in this analysis: IAEA086 and IAEA085 (International Atomic Energy Agency, Vienna, Austria) and NIES13 (National Institute for Environmental Studies, Onagawa, Japan). These SRMs were selected as the certified concentrations of $MeHg^+$ span several orders of magnitude from 258 µg/kg (IAEA086) to 22,900 µg/kg (IAEA085). The SRMs are pre-dried and homogenized, although prior to analyses a subsample of each SRM was freeze-dried in our laboratory to ensure low moisture content using a Labconco FreeZone 6 Liter (Labconco, Kansas City, Missouri, USA).

Five to10 mg of each SRM were weighed into 40mL Trace Clean© amber vials (VWR, Radnor PA). Digestions were done in triplicate for each SRM/technique, resulting in 9 digests for each digestion method (36 total).

6.3.1 Potassium hydroxide/methanol digest

This method has been reported elsewhere (e.g. Woshner et al. 2008) and has been used with various modifications including microwaves (e.g. Ramalhosa et al. 2001). Briefly, 25 grams of potassium hydroxide (KOH) pellets were slowly mixed into 100mL of swirling methanol to create a 25% KOH solution. Then ten mL of this solution was added to each amber vial containing SRMs. The vials were then vortexed and placed in a dark location for 36 hours. Following digestion, 20mL of methanol was added to the solution to bring the final volume to 30mL.

6.3.2 Nitric acid/water digest

A solution of 30% (v/v) nitric acid was made by diluting stock HNO_3 with milli-Q water. Three different volume nitric acid digestions were attempted: (a) 10mL, (b) 5mL, and (c) 2.5mL of 30% HNO_3 were added to amber vials containing SRMs. The samples were vortexed for 10 seconds and then placed in a hot water bath at 65°C for 36 hours, making sure that the height of the water was higher than the level of the nitric acid but not submerging the entire vial. The water bath was covered with an opaque cloth to prevent photodegradation of the samples. Following digestion, (a) 20mL, (b) 10mL, and (c) 5mL of milli-Q water was added to each digestion to bring the final concentration of nitric acid to 10% v/v. Following the addition of water, the samples were vortexed again for 10 seconds and placed into a dark drawer.

6.3.3 Brooks Rand/MERX

Methylmercury was analyzed using the Brooks Rand Model III detector in conjunction with the MERX autosampler system (Brooks Rand, Seattle, Washington, USA) using a 7 point calibration curve as in Taylor et al. (2011). The method below is slightly modified from EPA Method 1630 in order to incorporate the autosampler. Forty mL glass vials were filled to near the brim with milli-Q water. Next 300 µL of acetate buffer with antifoaming agent (Brooks Rand, Seattle, Washington, USA) was added to each vial. Thirty µL aliquot of digest was pipetted into a glass vial and each digest was run in duplicate. An ethylating reagent consisting of 2% sodium tetraethylborate ($NaBEt_4$) in 2%KOH/water was added and then quickly the remainder of the vial headspace was filled with milli-Q water from a squirt bottle (in order to prevent bubbles). The septa caps were then screwed on tightly to the vials. The vials were placed in racks on the MERX autosampler, and pulled sequentially into a bubbler, GC column, and detector (EPA Method 1630) as outlined in Harley et al. (2015).

6.3.4 Steller sea lion hair samples

Steller sea lion hair samples were selected from archived samples collected in 2015 from Agattu Island, AK. Permits obtained for capture and sampling are presented in Chapter 3. Hair samples were

washed 3x using Triton-X detergent and freeze dried to remove moisture. Total mercury concentrations in hair were obtained using a DMA-80 using the method reported in Castellini et al. (2012).

6.3.5 Statistics and graphics

In order to analyze percent recoveries from our different digestion methods, we utilized a general linear model (glm) with a Poisson distribution. We then used Tukey post-hoc contrasts to compare digestion methods. All statistics were done in the R programming language (R Core Team 2014), and all graphical representations were generated using the ggplot2 package (Wickham H. 2009).

6.4 Results/Discussion

Using a glm, we compared the nitric acid (30mL) digest with the KOH/methanol digest (30mL). The nitric acid had significantly better recoveries ($p<<0.01$ using Tukey post-hoc comparison, Figure 6.1). The KOH/methanol digestion had a mean percent recovery of 81%, while the nitric acid digestions had mean percent recoveries of 97% (30 mL), 107% (15 mL) and 115% (7.5 mL) (Figure 6.2a). All nitric acid digestions yielded significantly different percent recoveries than the KOH/methanol digestion. KOH/methanol digestions also had high variation between technical replicates – the mean percent relative standard deviation (RSD) was 22% for KOH/methanol (Figure 6.2b), while for nitric acid the mean percent RSD was 14% (30 mL), 10% (15 mL), and 4%(7.5 mL).

Within nitric acid digestions the percent recoveries appeared to increase with decreasing digestion volume, however only the 30mL nitric acid digestion was significantly different than the 7.5mL digestion volume ($p=0.002$, Figure 6.2a). Similarly, mean percent RSD significantly decreased with decreasing digestion volume, as all digests were significantly different from each other ($p <0.05$ using Tukey post-hoc comparisons, Figure 6.2b). The percent recovery for the smallest nitric acid digestion (7.5 mL) was high (115%), and while still acceptable by internal laboratory standards we would suggest for samples where there is ample quantity using a higher digestion volume. Smaller digests could undoubtedly be optimized for samples with extremely low concentrations of $MeHg^+$ or samples with low mass (i.e. <

1mg), but for most analyses of piscivorous mammalian hair the larger digestion volumes (15 or 30 mL) will provide better results.

Based on the results of our SRMs, we decided to use the 30mL digestion volume for the sea lion hair samples. Mean concentration of $MeHg^+$ was 12,300 µg/kg and mean [THg] was 17,300 µg/kg. THg and $MeHg^+$ were highly correlated (Figure 6.3), and using a robust linear regression the slope of the line was determined to be 0.72. Wagemann et al. (1997) suggested the slope coefficient of a robust linear regression would provide a better estimator of mean $\%MeHg^+$ compared to arithmetic mean, although in this case there was little variance in the relationship between THg and $MeHg^+$ (R^2=0.95) so the arithmetic mean $\%MeHg^+$ (71%) was not drastically different than the average as estimated by the slope parameter (72%). These values are somewhat lower than estimates reported for humans , although $\%MeHg^+$ some values reported for other piscivorous mammals have been lower (i.e. 65-78%, Evans et al. 2000). Percent $MeHg^+$ in hair or fur is not commonly reported in marine mammals, and to our knowledge this is the first time this has been assessed in this threatened population of Steller sea lions. Percent $MeHg^+$ was not significantly associated with [THg], indicating that the $\%MeHg^+$ in these hair samples was relatively consistent across a wide range of [THg] values.

We have demonstrated the efficacy and simplicity of this nitric acid digestion procedure, and shown the superiority of an acidic digestion as compared to a basic digestion for the analysis of mammalian hair. We feel it is valuable to assess the $\%MeHg^+$ in mammalian hair samples, especially in systems where there might be confounding factors of inorganic Hg exposure (i.e. dental amalagams or industrial exposure in humans). By assessing the $[MeHg^+]$ as well as the $\%MeHg^+$ in hair samples it is possible to address sources of exposure, which may shed light on occupational risk assessment (humans) or feeding ecology (wildlife).

6.5 Figures

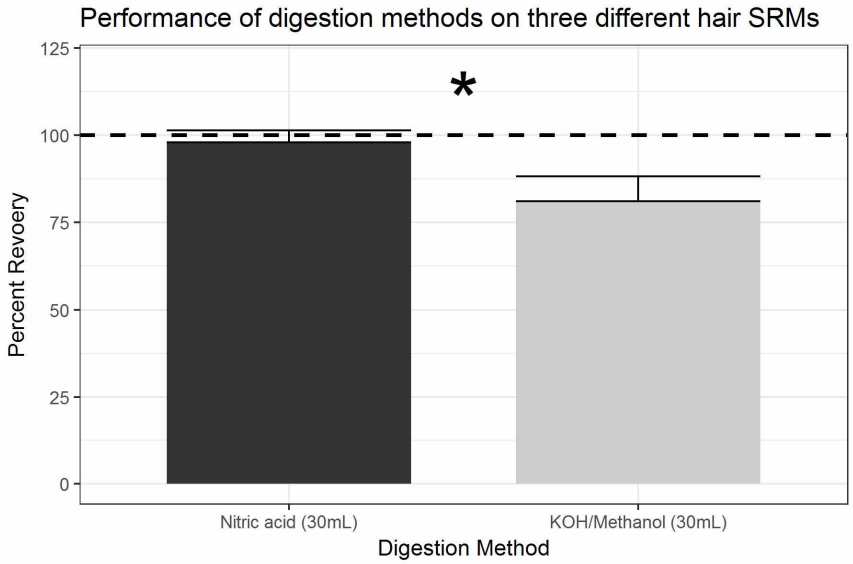

Figure 6.1 – A comparison of nitric acid versus potassium hydroxide digestions of hair SRMs using a 30mL digestion volume. SRMs used were IAEA85, IAEA86, and NIEHS13 (hair matrices). Percent recoveries were significantly different using a general linear model and Tukey post-hoc comparisons ($p<<0.01$).

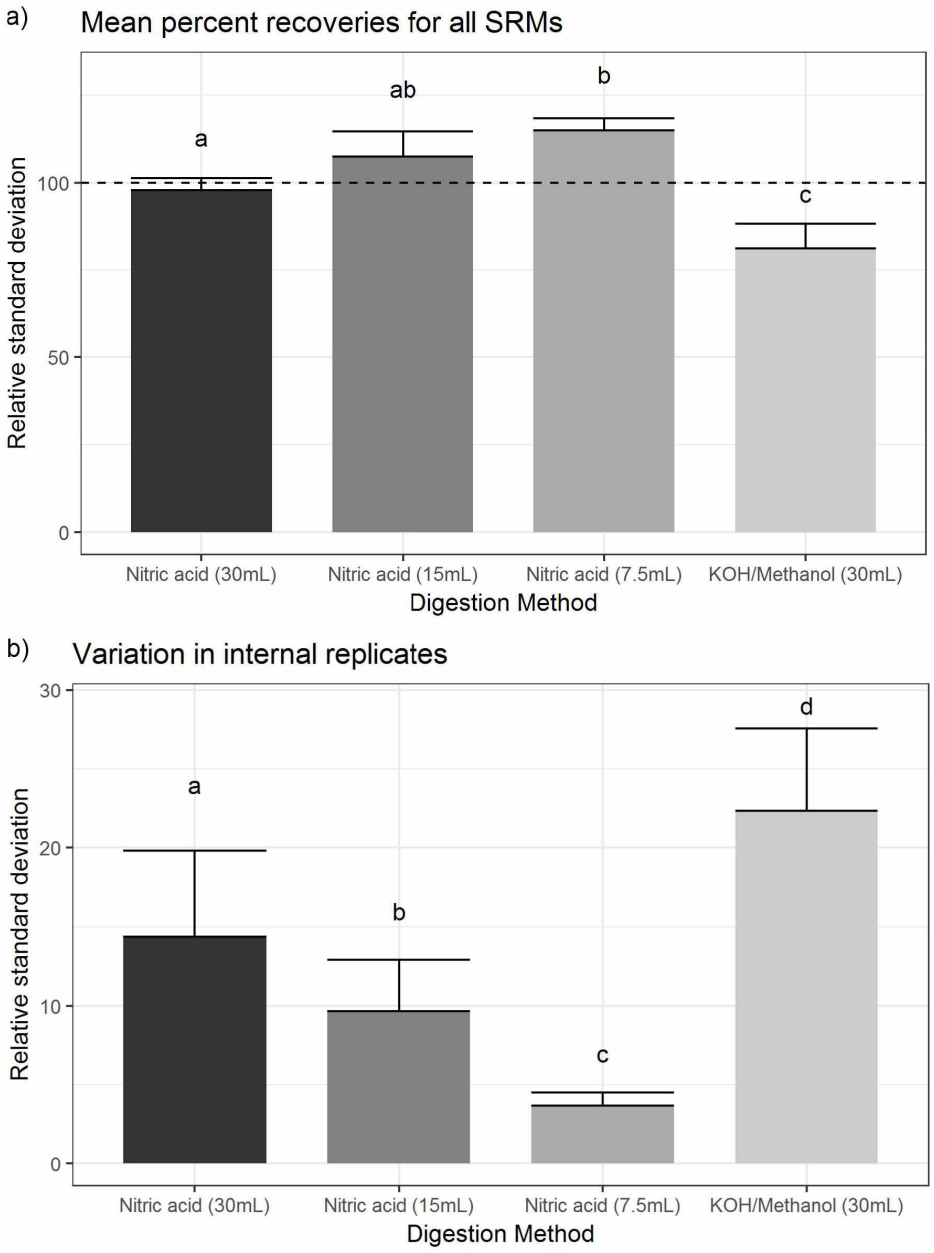

Figure 6.2 –a) shows mean percent recovery (error bars represent standard error) for each digestion method. The dashed line represents 100% recovery. b) shows the mean percent relative standard deviation in internal replicates (aliquots drawn from the same digestion) for each digestion method. For both plots, the letters above each digestion method indicate the statistical groupings as indicated by the Tukey post-hoc test (significant at the α=0.05 level).

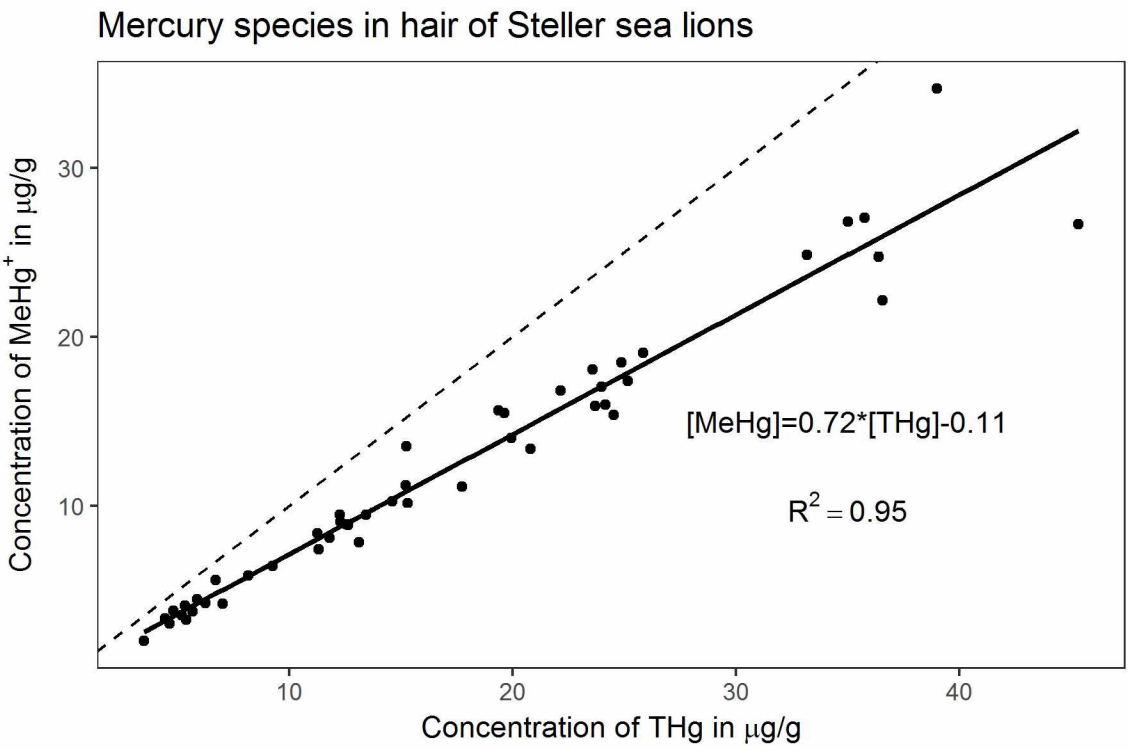

Figure 6.3 – Hair mercury concentrations for Steller sea lion hair. Using a robust linear regression, the slope was determined to be 0.72, thus the average percent $MeHg^+$ of THg was 72%. The dashed line represents 1:1 correspondence between $MeHg^+$ and THg (100% $MeHg^+$).

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